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Extracellular matrix metalloproteinase inhibitor (EMMPRIN), a plasma membrane glycoprotein identified originally in carcinoma cells, is responsible for inducing peritumoral fibroblasts to produce matrix metalloproteinases (MMPs), thereby enhancing cancer invasion and metastasis. Using in situ hybridization and immunohistochemistry, we have identified EMMPRIN in breast cancer tissue. We have characterized the human EMMPRIN gene and have noted a high degree of conservation with the mouse gene. In the 5' flanking region, three SP1 and two AP2 sites were identified in the promoter region. The effect of transfecting human breast cancer cells with EMMPRIN cDNA was explored. Expression of EMMPRIN cDNA by MDA-MB-436 cancer cells resulted in marked increase in tumor growth and invasiveness after tumor cell injection into mammary tissue of nude mice. Using the phage display system, crosslinking and ligand affinity chromatography, MMP-1 binding to EMMPRIN was identified suggesting the novel possibility that EMMPRIN binds MMP-1 to the tumor cell surface. The effect of EMMPRIN on endothelial secretion of MMPs was explored in vitro; EMMPRIN enhanced endothelial cell production of stromelysin-1, collagenase, and gelatinase A. These data support an important role for EMMPRIN in enhancing cancer dissemination. The regulation of EMMPRIN expression in malignant tissue remain to be examined.

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Stanley Zucker 12/22/98

PI - Signature Date

(4) TABLE OF CONTENTS

(5) INTRODUCTION

The purpose of this grant has been to determine the role of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) in breast cancer invasion and metastasis. EMMPRIN is a glycoprotein identified on the plasma membrane of cancer cells which induces fibroblasts to produce Matrix Metalloproteinases (MMPs) (1). Originally known as Tumor Collagenase Stimulating Factor (TCSF) because it was described as a stimulator of interstitial collagenase (MMP-1) production by fibroblasts, TCSF was subsequently demonstrated to also induce target cells to synthesize gelatinase A and stromelysin-1, hence the name change to EMMPRIN to reflect induction of synthesis of other MMPs.

EMMPRIN (TCSF), was initially purified from the plasma membranes of cancer cells by Biswas et al. and identified as a 58 kDa glycoprotein. EMMPRIN had no mitogenic activity and therefore differs from most well characterized cytokines, such as IL-1, TNF, and TGF-β. Monoclonal antibodies raised against EMMPRIN were used to purify and characterize this membrane glycoprotein (2). The E11F4 monoclonal antibody also inhibited the biological activity of EMMPRIN, thereby proving that EMMPRIN was the effector molecule. Following development of specific monoclonal antibodies, EMMPRIN was identified by immunohistochemistry in the cell membranes of malignant epithelial cells from tumor specimens of human lung cancer and bladder cancer (3). Recent reports from other laboratories have confirmed the stimulatory effect of human breast and bladder cancer cells on production of MMPs by host fibroblasts (3, 4).

The main thesis of this grant is that breast cancer cells produce EMMPRIN which then induces peri-tumoral fibroblasts to produce the MMPs (collagenase, gelatinase A, and stromelysin-1) required for cancer dissemination. The biologic role of EMMPRIN in cancer was not initially appreciated because tumor cell lines grown in vitro generally produce high levels of MMPs, thereby suggesting that tumor cell production of MMPs was responsible for cancer invasion and metastasis. Fibroblast production of MMPs was relegated to a secondary role. In retrospect, this confusion seems to have been brought about because cancer cells that develop into immortalized cells in tissue culture generally produce high levels of MMPs. Cancer cells incapable of producing MMPs in vitro seem to be at a disadvantage in terms of developing into immortalized cell lines. Based on these observations, we postulate that high MMP production is a selection factor for enhanced cell growth in vitro. Hence, although the vast majority of cancer cells within a tumor do not produce MMPs in high levels, the few cells that produce MMPs appear to have a growth advantage in tissue culture. These data may explain the lack of enthusiasm for a role for EMMPRIN in cancer dissemination until it was demonstrated that the MMP producing cells within a tumor are primarily host fibroblasts rather than cancer cells.

Considerable evidence has been presented in the past 4 years to support the Biswas concept that cancer cells signal fibroblast to produce MMPs (1). In situ hybridization studies of human breast cancer tissues have shown that the cDNA for matrix metalloproteinases (stromelysin-3, gelatinase A, interstitial collagenase, MT-MMP) is localized to fibroblasts surrounding the tumor rather than to the tumor cells themselves (5, 6). Immunohistochemical studies using monoclonal and polyclonal antibodies, however, have identified gelatinase A in breast cancer cells suggesting that the tumor cells may bind this MMP to their cell surface (7-9). Interpretation of these data has led to the conclusion that normal host fibroblasts produce much of the MMPs that the cancer cells utilizes during invasion (10).

Using immunohistochemistry, we have demonstrated the selective localization of EMMPRIN on the surface of malignant cells in human breast cancer tissue (11), further suggesting that this factor may provide the missing link to explain the observation that peritumoral fibroblasts are the major producers of MMPs.

Based of the potential importance of EMMPRIN in regulation of MMP activity during tumor cell invasion, we have studied EMMPRIN at the molecular and physiologic level. Following determination of the nucleotide sequence of the cDNA for human EMMPRIN (1), it

was recognized that EMMPRIN is homologous to proteins of the Ig superfamily (basigin, neurothelin, OX-47, M-6) (12, 13) which have been identified in arthritis and embryonic epithelial/stromal interactions; the function of these proteins was not explored in these latter studies, but a function similar to EMMPRIN would be appropriate in these situations.

SUMMARY OF IMPORTANT PUBLICATIONS IN 1998 THAT ARE RELEVANT TO THIS GRANT

1) Association of EMMPRIN with integrins at the cell surface

Recent studies examining the formation of complexes between integrins and other cell surface proteins using monoclonal antibody and cross-linking approaches have demonstrated that EMMPRIN/basigin is associated with $\alpha 3\beta 1$ and $\alpha 6\beta 1$, but not $\alpha 2\beta 1$ or $\alpha 5\beta 1$ integrins. Immunofluorescent studies showed that EMMPRIN co-localizes with $\alpha 3\beta 1$ in cell-cell contacts and may interact with one another in a receptor-ligand fashion or in a lateral fashion (14). Chen has recently shown that EMMPRIN is localized along with several other proteases to invadopodia of breast carcinoma cells. This observation is of particular interest to us since new data from our lab suggests that EMMPRIN not only stimulates MMP-1 production but also binds MMP-1. Therefore, this localization of EMMPRIN to invadopodia would also concentrate MMP-1 at the invasive region of the tumor cell surface.

2) Structure of the mouse EMMPRIN/basigin gene

It is now evident that there is a family of molecules related to EMMPRIN. A recent study of rat synaptic membranes reveals two major Ig superfamily proteins, gp65 and gp55, that are closely related but not identical to OX47, a rat-EMMPRIN like protein. Thus, in the rat, there are at least 3 proteins closely related to EMMPRIN (15).

(6) BODY OF ANNUAL REPORT

Experimental Results (the original timetable for these tasks is listed in parentheses)
Accomplishments achieved in years 1-3 (12/94 to 11/97) and year 4 (12/97 to 11/98) are identified separately.

Task (1). Identify the cellular localization of EMMPRIN (TCSF) and MMPs in human breast cancer tissue.

(1a) Obtain tissue samples from patients with various forms of breast cancer (24 months):

12/94 to 11/97- Breast tissue samples were obtained from 87 women with various forms of breast cancer and women with benign breast disease.

12/97 to 11/98- Breast tissue samples were obtained from an additional 12 patients. This number of samples has been sufficient to perform the experiments initially planned.

(1b) Immunolocalization of EMMPRIN and MMPs in human breast cancer tissue using specific antibodies to EMMPRIN to determine epithelial:mesenchymal contributions (36 months):

12/94 to 11/97- Using immunohistochemical techniques and our original E11F4 monoclonal antibody to EMMPRIN, we characterized the cellular localization of EMMPRIN as compared to the localization of gelatinase A in breast cancer. Tumor sections obtained from 28 women with breast cancer were examined. In all cases of invasive ductal cancer, antibodies to EMMPRIN reacted strongly with invasive cancer cells with intense staining of the plasma membrane and less intense staining of cytoplasm. In comparison with cancer cells, normal ducts within the tumor specimen demonstrated similar staining with anti-EMMPRIN antibody. EMMPRIN immunostaining was intense in both early and advanced stages of invasive breast cancer, as well as in situ breast carcinomas. Moderate EMMPRIN staining of breast ducts and acini was noted in breast tissue obtained from biopsies of patients with benign breast disease and normal breast tissue (obtained from mammary reduction surgery). Colored photos were displayed in the 1996 report.

Limitations in quantifying EMMPRIN antigen concentrations in human tissue using the E11F4 monoclonal antibody could not be ruled out. These data suggested that EMMPRIN may have a function in embryonic development or maintenance of normal breast tissue, as well as the malignant process. We propose that in physiologic processes, the presence of an intact basement membrane separating the normal/benign epithelium from underlying stromal fibroblasts limits access of epithelial cell EMMPRIN for induction of MMP production by stromal fibroblasts through a cell-cell contact related mechanism. In contrast, in carcinomas the epithelial basement membrane is fragmented, thereby permitting epithelial cancer cells to migrate into the stroma, make direct cell contact with fibroblasts, and stimulate fibroblast synthesis of MMPs. The enhanced production of MMPs by peritumoral fibroblasts then leads to degradation of the stroma (including the basement membrane), thereby enhancing the invasive/metastatic process of cancer cells.

12/97 to 11/98- The discrepancy between our results on immunohistochemistry and in situ hybridization (see below) have led us to repeat our earlier immunohistochemical studies using a different monoclonal antibody to EMMPRIN. In this study we used a recently developed mouse monoclonal antibody directed against EMMPRIN (see below) for immunohistochemical localization of EMMPRIN. Fresh frozen tissue from 10 women with breast cancer and 2 specimens of fibroadenoma of the breast were examined. EMMPRIN protein was prominently displayed in malignant breast tissue on both the surface of tumor epithelial cells and fibroblast-like stromal cells; occasional endothelial cells displayed spotty labeling on their cell membranes. In carcinomas there were also fine positive vesicles in the connective tissue close to tumor cells suggesting that EMMPRIN could be released as membrane components by tumor cells. EMMPRIN was frequently confined to the apical cell membrane. In normal or benign cells, EMMPRIN was also detected as a weak label at the basal pole of the epithelial cells. In tumor clusters, EMMPRIN was highly expressed in all

malignant cells with more intense staining on the cells located at the periphery of well differentiated tumor nests (Figure 1). Colocalization of EMMPRIN with MMP-2 was examined. In all carcinomas, we observed that the presence of EMMPRIN in/on stromal cells was concomitant with the detection of MMP-2 in the same cells. Using double labeling of immunofluorescence, we found that the stromal positive staining of EMMPRIN was not necessarily associated with the absence of type IV collagen around tumor clusters suggesting that basement membrane integrity is not a limiting factor for EMMPRIN diffusion from the producing cell. Since previous studies have demonstrated that stromal cells did not express EMMPRIN mRNAs, it is very likely that EMMPRIN is release or shed as vesicles from the membranes of cancer cells and then binds to stromal cells via a receptor. Taken together, our results showed that EMMPRIN is an important factor in tumor progression by causing tumor-associated stromal cells to increase their MMP production, thus facilitating tumor invasion and neoangiogenesis.

(1.b.1) Production of monoclonal antibodies to EMMPRIN

12/94 to 11/97- Production of monoclonal and polyclonal antibodies to recombinant EMMPRIN for use in ELISAs. Eight BALB/c mice were immunized by the intraperitoneal injection of recombinant EMMPRIN (purified from CHO cell homogenates transfected with EMMPRIN cDNA). In spite of the development of high serum titers of antibodies against the immunogen in mice, antibodies adequate for ELISA were not produced. The problem with the initial 20 mouse myeloma clones developed was that all of the antibodies were of the IgM type and reacted nonspecifically with other proteins as demonstrated by Western blotting.

12/97 to 11/98- To circumvent these problems, we used EMMPRIN purified from human lung cancer cells (LX-1) rather than recombinant EMMPRIN as the immunogen. EMMPRIN antigen injections (100 ug) in mice were performed on a 2 week schedule and mice were sacrificed after 8 weeks. This work was done in collaboration with Chemicon Corp. (Drs. Dale Dembro and Alex Strongin, San Diego, CA). Antibodies to EMMPRIN in mouse serum were present at a titer of 1:10,000 using native antigen rather than recombinant antigen. Spleen myeloma cells fusions growing in splenocyte conditioned media resulted in the production of 63 positive IgG producing wells. The 15 clones with the highest antibody titers as detected by EIA (20 ng EMMPRIN per well) were subcloned with 8 active clones identified after two subcloning procedures. All 8 clones produced distinct, broad bands (a result of extensive glycosylation) at ~53 kDa on Western blotting with EMMPRIN (Figure 1); a second band at 28 kDa representing either non glycosylated EMMPRIN or a degradation product of EMMPRIN was also noted. Non-specific cross reactivity with other proteins was not a problem. Two of these monoclonal antibodies have been characterized more fully and have been shown to be effective for Western blotting and immunohistochemistry (see Figure 1). We have used these antibodies to screen for cells expressing high levels of EMMPRIN antigen on their plasma membranes.

(1.b.2) New Aim: Production of monoclonal antibodies to Membrane Type (MT1)-MMP

12/94 to 11/97- Since the description of MT1-MMP in 1994 (16, 17), it has become apparent that this membrane bound MMP is important in cancer because it functions to activate progelatinase A in the pericellular environment. We propose that following the induction of progelatinase A synthesis by EMMPRIN, MT1-MMP is required for activation of progelatinase A. As with most MMPs, MT1-MMP has been described by in situ hybridization as being expressed in tumoral fibroblasts rather than epithelial cancer cells (18). Since our previous study demonstrated that EMMPRIN treated fibroblast secreted activated as well as latent gelatinase A (19) , this data suggested that EMMPRIN may also be responsible for inducing the synthesis of MT1-MMP. Based on these observations we have developed high titer monoclonal antibodies that react with MT1-MMP by injecting recombinant human MT1-MMP (fusion protein with GST) lacking the transmembrane domain (protein produced in bacteria) into Balb/c mice. Following fusion of mouse spleen cells with immortalized myeloma cells, antibody producing clones were selected. Six of these monoclonal antibodies

demonstrate high titer efficiency as detecting antibodies for immobilized MT1-MMP. In these studies, we became aware that all of these monoclonal antibodies were reactive only with secreted forms of MT1-MMP lacking the transmembrane domain. These antibodies were not reactive with cell-bound MT1-MMP and hence, are not useful for immunochemistry or ELISAs of tissue homogenates. These antibodies, however, might be useful for identifying secreted/shed forms of MT1-MMP in body fluids.

(1c) Develop an ELISA for EMMPRIN (TCSF) for use in quantifying antigen in breast tissue (6 months):

12/96 to 11/97- The development of an ELISA for EMMPRIN has been delayed until recently because of the absence of antibodies of sufficiently high titer and affinity for EMMPRIN. These improved antibodies are now available (see above). Purified EMMPRIN (100 ug) has recently been biotinylated and was used to determine which of the monoclonal antibodies described in Section 1.b. 1 functions best as a capture antibody in an ELISA. Each of the monoclonal antibodies has been immobilized in 96 well plates and after washing and blocking steps, biotinylated EMMPRIN was added for 1 hour. Streptavidin- alkaline phosphatase and substrate were used for color development. The results of these experiments indicated that of the 2 monoclonal antibodies tested, neither antibody functioned well in capturing EMMPRIN antigen in solution. Both of these monoclonal antibodies, however, functioned well as detecting antibodies; biotinylation of the antibodies did not interfere with function as detecting antibodies.

(1d) Quantify the EMMPRIN, gelatinase A, gelatinase B, and stromelysin-1 content of fresh tissue samples obtained from patients with breast cancer (36 months):

12/94 to 11/97- These studies have not been completed, to date, because we have not been successful in quantifying the concentration of EMMPRIN in tissue samples. Our ELISA has not been useful for tissue assay of EMMPRIN.

(1e) Identify mRNA for EMMPRIN (TCSF) in breast cancer tissue using in situ hybridization (48 months):

12/94 to 11/97- This task was completed ahead of schedule. To characterize and distinguish the cells producing EMMPRIN and gelatinase A in breast cancer, we employed in situ hybridization using radiolabeled RNA probes for EMMPRIN and gelatinase A (20). Surgical specimens were obtained from 22 women with breast cancer and from 7 women with benign breast disease (fibrocystic disease and fibroadenoma). The result of these studies was that EMMPRIN mRNA was detected by in situ hybridization in all carcinomas in both non invasive and invasive cancer cells and in pre malignant areas such as atypical hyperplasia of the breast. EMMPRIN mRNA and gelatinase A mRNA were both visualized in the same areas in serial sections in breast cancer, but were expressed by different cells with tumor cells expressing EMMPRIN mRNA and fibroblasts expressing gelatinase A mRNA. There was no correlation between EMMPRIN mRNA and the tumor size, grade of the tumors, the number of lymph node metastases, and the hormonal receptor status of the tumors. Normal mammary glands adjacent to cancer areas showed no EMMPRIN hybridization grains. By Northern blot analysis of tissue extracts, higher expression of EMMPRIN mRNAs in breast cancers was noted as compared to benign and normal breast tissue; however, the normal tissue expression was not negative as noted by in situ hybridization. The discrepancy noted between normal breast ducts staining positively for EMMPRIN by immunohistochemistry but negatively by in situ hybridization suggested differences in sensitivity of these techniques for EMMPRIN detection or differences in the rates of EMMPRIN turnover in normal versus malignant tissue (21).

Task (2). Identify important structural:functional relationships in the EMMPRIN (TCSF) molecule.

(2a) Determine whether post-translational processing is required for biological activity (12

months): **This task has been completed.**

12/96 to 11/97 - To circumvent problems related to the requirement for glycosylation of EMMPRIN for function of the protein, we stably transfected mammalian cells (CHO) with EMMPRIN cDNA. Posttranslational processing resulted in the production of EMMPRIN of molecular weight identical to native EMMPRIN from tumor cells i.e. ~58 kDa. We immunopurified the recombinant EMMPRIN after extraction from CHO cell membranes using monoclonal antibodies raised against native tumor cell EMMPRIN. When added to human fibroblasts in culture, the purified recombinant EMMPRIN was found to be active in stimulating production (2-5 fold) of fibroblast interstitial collagenase, gelatinase A, and stromelysin-1, but not TIMP-1 (22). Since non-glycosylated and partially glycosylated recombinant EMMPRIN were unable to stimulate MMP production, we conclude that post-translational processing is required for EMMPRIN activity.

(2b) Alter EMMPRIN (TCSF) by deletional mutation and site directed mutagenesis of cDNA and then analyze mutant proteins to determine the minimum amino acid sequences necessary for functional activity (36 months):

(2.b.1) **12/94 to 11/97**- We reconfirmed the identity of the EMMPRIN cDNA by showing that recombinant protein is recognized by the activity blocking monoclonal antibody (E11F4). Two immunoreactive bands were obtained corresponding to the forms previously noted (1). The bacterial recombinant proteins was also used to determine the approximate location of the epitope for E11F4, taking advantage of the lack of posttranslational modification that would interfere with such studies on the native, immunoaffinity-purified protein. Modified EMMPRIN expression plasmids were made containing deletion in four locations. XL-1 blue cells were transformed with the deletions expression pBluescript plasmids, and the expressed proteins were analyzed by SDS-PAGE and Western blotting with monoclonal antibody E11F4. All of the plasmids produced protein that is immunoreactive, except for the plasmid lacking immunoglobulin domain I. These results demonstrate that our cDNA encodes the protein which is reactive with our activity-blocking monoclonal antibody and that the antibody epitope exists in the extracellular immunoglobulin domain I. This, in turn, implies that the functional site of the metalloproteinase stimulatory activity of EMMPRIN is likely to be localized to sequences contained in the immunoglobulin domain I region.

(2.b.2) Characterization of the human EMMPRIN gene

As a result of our sequencing of keratinocyte cDNA for EMMPRIN and its strong homology with cancer cell EMMPRIN, and the identification of EMMPRIN in normal rabbit kidney cells, T lymphocytes, and erythrocytes, we considered the strong possibility that the difference between the high level of synthesis of EMMPRIN in cancer cells and the apparent low level synthesis in non malignant cells may be due to differences in the promoter region for EMMPRIN, specifically in the mixed response element category. This led us to undertake the sequencing of the entire human EMMPRIN gene as a priority goal.

cDNA hybridization have been used by other investigators to determine the chromosomal location of mouse EMMPRIN/basigin gene, mapping to chromosome 10.

12/96 to 11/98- We have recently reported the characterization of the human EMMPRIN gene. Using EMMPRIN cDNA, we isolated a >30 kbase cosmid clone containing the human EMMPRIN gene and confirmed the gene localization to chromosome 19p13.3. Using a fragment of the clone, S1 was performed to determine the transcriptional start site. The exon/intron boundaries were determined by genomic PCRs and sequencing. The gene fragment contains ~ 1kBase of sequence upstream of the transcription start site. This 5' flanking region does not contain TATA or CAAT boxes (figures presented in 1997 report). However, the start site falls within a CpG island. Also three consensus binding sites for SP1 and two for SP2 were found (23).

Elements in the proximal promoter region were conserved in human and mouse genes. The human and mouse genes have the unusual property that each Ig domain is not encoded by one exon, but by two. Also unusual in the EMMPRIN gene as compared to other Ig family

members is the fact that the downstream exon of the second Ig domain is a junctional exon encoding the transmembrane domain and part of the cytoplasmic domain as well. Most members of the Ig superfamily encode the Ig domain in a single, unshared exon.

These data lead us to postulate that when cells become transformed they might produce more SP1 and AP2 and those are the factors which bind to the EMMPRIN promoter, thereby enhancing the transcription and subsequent translation of EMMPRIN.

(2c) Design peptide antagonists and produce anti-functional monoclonal antibodies to further characterize the structure:functional relationship of the EMMPRIN molecule (48 months):

This task was not able to be completed within the time frame of this study. We have produced several monoclonal antibodies to EMMPRIN, but none of these has been demonstrated to have anti-functional activity.

Task 3. Explore the role of EMMPRIN (TCSF) in cancer dissemination using experimental models.

(3a) Compare the effect of transfecting breast cancer cells with cDNA for native versus mutant EMMPRIN in regards to altering cancer invasion and metastasis in an experimental model (48 months):

12/94 to 11/97- *Preliminary experiment:* The open reading frame for EMMPRIN cDNA was transfected into human breast cancer cell lines (MDA-MB-436 and MCF-7). Transfected cell lines were then injected into the mammary fat pad of 6-8 week old nude female mice at a cell concentration of 1 X 10^6 per animal. Palpable tumors (1-4 cm in diameter) were detected after 3 months in 2/4 mice injected with EMMPRIN cDNA- transfected MDA-MB-436 cells and in 3/4 mice with mock transfected MDA-MB-436 cells. Histology of the breast cancers was as anticipated indicating that the morphologic phenotype had not changed. This experiment was subsequently refined in 1997 as described below.

Additional experiments: To ascertain that transfected tumor cells are expressing EMMPRIN protein in high concentration, we took advantage of the recent observation that the green fluorescent protein (GFP) of the jelly fish Aequoria victora retains its fluorescent properties when recombinantly expressed in eukaryotic cells (24). This 29 kDa protein can then be used as a powerful marker for gene expression in vivo (25). To this end, we expressed EMMPRIN cDNA along with the GFP reporter cDNA as a fusion gene controlled by a CMV promoter in pcDNA3 expression vector. The GFP-EMMPRIN fusion protein was expressed in the plasma membrane of transfected COS-1 cells as documented using fluorescent microscopy, thus indicating that GFP is transported to the plasma membrane along with the EMMPRIN protein. A second plasmid was produced in which GFP and EMMPRIN are controlled individually by separate CMV promoters. A control plasmid containing GFP alone was also produced. We documented EMMPRIN expression in transfected cells by fluorescent microscopy and immunoblotting of cell lysates.

MDA-MB-436 human breast cancer cells were transfected with GFP cDNA and EMMPRIN/GFP fusion cDNA. Stable cell lines were selected using G-418. After 5 weeks, stably transfected cells were examined by Northern blotting. The expression of EMMPRIN mRNA in EMMPRIN transfected MDA-MB 436 cells was approximately 4 fold higher than in vector transfected cells. 1 x 10^7 tumor cells derived from these transfected cell lines were then injected into mammary tissue of 4 month old nude female mice. Mice were examined weekly thereafter for tumor size measurements.

Results: EMMPRIN/GFP transfected mice (7/10) developed breast tumors at the site of mammary injections that grew to 1.4-2.3 cm diameter within 7-12 weeks; intra-abdominal metastases were extensive in most mice (retroperitoneal, perigastric, sub diaphragm, kidney, etc.). These tumors were highly vascularized with numerous grossly visible vessels entering the tumor mass from normal tissue. GFP expressing tumors were green in color when examined by fluorescent light. Lung metastases were not observed in any of the groups. By

comparison, the GFP (only) transfected tumor cells produced small, slow growing, poorly vascularized tumors (3/4 mice) that reached a diameter of ~2-3 mm in 12 weeks (not visible until autopsy).

12/97 to 11/98- These experiment have been repeated in order to determine the reproducibility of these observations. Groups of 10 nude female mice (5 weeks of age, younger than our 1997 experiment) have been injected with 5 X 10^6 transfected MDA-MB-436 cells (lower cell number) into mammary tissue as described above. Tumor formation and metastases were recorded during 12 weeks of observation. Major differences between groups were noted. The tumors derived from the EMMPRIN-GFP cDNA transfected MDA-MB-436 tumor cell injections grew much more rapidly and all 10 mice expired within 12 weeks; intra-abdominal metastases were frequent in these mice. In contrast, injection of the GFP cDNA alone transfected tumor cells into mice resulted in tumors that grew much more slowly; none of these mice expired and no intra-abdominal metastases were noted by termination of the experiment at week 12 (Figure 2). Blood vessels leading to the tumor were considerably more extensive on gross observation of mice in the EMMPRIN-GFP transfected MDA-MB-436 group. Additional studies are underway to examine the extent of tumor neoangiogenesis in these mice using antibodies specific for endothelial cells (CD31 or Factor VIII). These experiments will be completed during the extension of this grant in 1999.

These experiments suggests that: 1) transfection with EMMPRIN cDNA resulted in more rapid tumor growth, intra-abdominal metastases, and extensive tumor neovascularization; and 2) in the background of this less malignant transfected phenotype, expression of EMMPRIN cDNA resulted in more invasive and locally metastatic cancer cells.

We have examined a second breast cancer cell line to determine whether the EMMPRIN transfection effect is reproducible in other cell lines. Transfection with EMMPRIN cDNA into MDA-MB-435 cells (widely metastatic cell lines supplied from Georgetown University) has been performed as described above for MDA-MB-436. To simplify the interpretation of results, the GFP cDNA was not be included in this experiment. Based on immunohistochemistry, we have identified 2 clones of high EMMPRIN-producing cells and 2 clones of lower EMMPRIN producing cells. Eight mice in each group, which also included the wild-type cells, were injected into the mammary fat pad with $5X10^6$ transfected cells. Tumor growth and metastasis of these cells as compared to non-transfected and vector-transfected cells are in the process of being examined. These experiments will be completed during the extension of this grant into 1999.

(3b) Analyze human peritumoral fibroblast response to EMMPRIN (TCSF) in vitro (36 months):

(3.b.1) In a collaborative study with Dr. Nabeshima et al. we have investigated the expression of EMMPRIN in human brain tumors, especially astrocytic tumors. Both normal brain and astrocytic tumors expressed EMMPRIN at the mRNA (Northern blotting) and protein (immunoblotting) levels, but expression levels were higher especially in high grade astrocytic tumors. Localization was quite different between normal and tumor tissue. In the normal brain only vascular endothelial cells were EMMPRIN-positive, whereas in the tumors the cancer cells were positive and the endothelium was negative (26). EMMPRIN positivity in endothelium was present only in the brain which is consistent with localization in the blood brain barrier as previously reported for Basigin (murine EMMPRIN) in mice.

In vitro co-culture of EMMPRIN-positive glioblastoma cells and brain-derived fibroblasts resulted in increased gelatinase A production as compared with control, and this stimulatory effect was inhibited by anti-functional anti-EMMPRIN antibody (EIIF4). This data is consistent with the involvement of EMMPRIN in tumor invasion and angiogenesis since brain-derived fibroblasts are present in perivascular tissue (26).

(3.b.2) Evaluate more directly the mechanism of action of EMMPRIN in terms of cell surface receptors, intracellular messengers, and mechanism of inducing MMP gene expression.

12/96 to 11/97- Based on additional information available about EMMPRIN since 1994, it would appear that identifying the receptor for EMMPRIN on fibroblast target cells is a priority in understanding the mechanism of action of EMMPRIN and in determining whether tumor derived fibroblasts are more responsive to EMMPRIN's stimulatory effect than non activated fibroblasts. Based on these considerations, we have performed receptor binding studies with EMMPRIN.

Cross linking- Human HFL (lung fibroblasts) were metabolically labeled with ^{35}S methionine (200 uCi/ml) for 5 hours. Cells were washed thoroughly to remove unincorporated isotope. Purified human EMMPRIN (100 nM) was added to cells for 1.5 hours. The cross-linking reagent, 0.5 mM DTSSP (Pierce Co.) was added for 30 minutes at 23° C. The cells were then washed thoroughly and cells were lysed in PBS buffer with 0.1% triton X-100 detergent. The cell lysate was incubated with anti-EMMPRIN antibody (E11F4) overnight and then added to protein A agarose beads for 1 hour. The beads were then washed six times with PBS and boiled with 1x sample buffer for 5 minutes. The samples were loaded on a 10% SDS-PAGE and exposed to XRay film. The results demonstrate the binding of EMMPRIN to a ~57 kDa and a ~42 kDa protein on fibroblasts.

We next examined the binding of EMMPRIN to fibroblast membranes. Membrane extracts from human fibroblasts were loaded on an EMMPRIN (0.5 mg) affinity column (Carbolink or Aminolink column, Pierce) for 30 min. After washing the column with PBS, the protein was eluted from the affinity column with elution buffer and the samples were analyzed by SDS-PAGE followed by silver staining. A single protein of 58 kDa was identified. These results support the concept that fibroblasts contain a binding protein (receptor) for EMMPRIN that has a mass of ~58 kDa. Based on this data, we will now be able to compare quiescent fibroblasts versus tumor derived fibroblasts for their ability to bind to EMMPRIN. These results should coincide with the comparison of the biologic inducing effect on MMP synthesis of EMMPRIN using tumor fibroblasts as planned in the initial grant.

12/97 to 11/98- We have used the T7Select Phage Display System (Novagen) to identify EMMPRIN binding proteins. We first made a cDNA library using poly(A) + RNA from human fibroblasts known to be sensitive to stimulation by T7Select vector and incubated with T7 packaging extract. Host cells (BLT5403) were then infected with the phage and prepared for biopanning. A solution of purified EMMPRIN (5 mg/ml) was used to coat 24-well plates. After washing with buffer, incubating with blocking solution, and re-washing with water, the wells were incubated with phage lysate and washed again to remove unbound phage. The bound phage were then eluted from the plates with SDS solution, incubated with fresh host cells and lysed for the next round of panning. Five rounds of biopanning were carried out and the final lysate used for plaque assay, PCR amplification and sequencing.

After the five rounds of panning described above, eight clones were obtained. Four of the inserts were of identical size and have been partially sequenced. All four have identical sequences and correspond to a portion of the human interstitial collagenase (MMP-1) sequence. This was an unexpected result but suggests the novel possibility that EMMPRIN binds MMP-1 to the tumor cell surface in similar fashion to the binding of gelatinase A by integrin alphaV-beta3 (27) or TIMP-2:MT1-MMP complex (28).

(3.c) <u>New Task</u>: <u>Effect of EMMPRIN on production of MMPs by endothelial cells</u>:

In the four years since this grant was written, it has become apparent that tumor angiogenesis plays an important role in the progression of cancer. An obvious question to be addressed is whether the tumor cells produce additional factors besides VEGF that would enhance tumor angiogenesis. Based on our demonstration that EMMPRIN produced by cancer cells stimulates fibroblasts to produce MMPs, we reckoned that EMMPRIN may have a similar effect on endothelial cells.

12/96 to 11/98- To address this question we have incubated EMMPRIN with endothelial cells to determine the effect on MMP production. EMMPRIN purified from stably transfected

CHO cells also stimulates production of stromelysin-1, interstitial collagenase, gelatinase A, and TIMP-1 by human umbilical vein endothelial cells cultivated in vitro (Figure 3). By comparison, VEGF had a greater stimulatory effect than EMMPRIN on HUVEC synthesis of interstitial collagenase and TIMP-1, but not gelatinase A or stromelysin-1 (29). We concluded that EMMPRIN plays a role in the early phase of tumor angiogenesis by inducing the degradation of endothelial basement membrane. We propose that in vivo, direct contact between circulating tumor cells and endothelial cells lining blood vessels at organs distant from the primary tumor may facilitate tumor cell penetration of the subendothelial basement membrane during metastasis (30). In this scenario, tumor cell EMMPRIN induces endothelial cells to secrete MMPs that subsequently facilitate subendothelial basement membrane degradation (Figure 4); tumor cells then are able to migrate through the rents in the blood vessel wall (30).

Our hypothesis that EMMPRIN is a positive factor in tumor angiogenesis is supported by our recent studies demonstrating more vascularized tumors produced by EMMPRIN-transfected breast cancer cells (see Task 3).

Task 4 (completed) added to original grant application (see 1996 annual report)
Human Keratinocyte EMMPRIN.

12/95 to 11/96- Although initial studies suggested that EMMPRIN is not present in significant amounts on many types of normal adult human cells (2, 3), it subsequently became apparent that EMMPRIN is identical to human basigin and M6 antigen, (12, 13) and is expressed in some physiologically active epithelia during embryonic development, as well as tumor cells. Since keratinocytes have previously been shown to stimulate MMP production by fibroblasts (31) and since epithelial-dermal interactions are important in preserving and repairing skin structures, we sought evidence for the presence of EMMPRIN in keratinocytes. We found that human keratinocytes express EMMPRIN at their cell surface in vivo and in vitro and synthesize EMMPRIN in culture, albeit at a lower level than tumor cells. On characterization of EMMPRIN cDNA obtained from a keratinocyte cDNA library, we found that the deduced amino acid sequence was identical to that of tumor cell EMMPRIN (32). These cDNAs share a common region of 1459 nucleotide residues that differ in only 7 of these residues, only two of which are in the open reading frame and which result in no differences in the amino acid sequence of EMMPRIN. The significance, if any, of the polymorphism in the cDNA sequence of the two clones is unclear. We concluded that human keratinocytes produce EMMPRIN. A role for EMMPRIN at epithelial dermal junctions in tissue repair during wound healing seems highly plausible. Taken together, these data suggest that the function of EMMPRIN is under strict regulation in normal tissues, but this control mechanism may go awry in cancer.

(7) CONCLUSIONS

EMMPRIN (TCSF) is a plasma membrane glycoprotein that is present on the surface of breast cancer cells, and is responsible, in part, for the elevated levels of MMPs in peritumoral fibroblasts and endothelial cells. EMMPRIN requires post-translational processing (glycosylation) for its ability to stimulate production of MMPs by target fibroblasts. Transfection of EMMPRIN cDNA into CHO cells resulted in the production of a glycosylated functional protein of similar molecular weight to native EMMPRIN (58 kDa) that was localized to the plasma membrane. EMMPRIN mRNA is expressed in benign and malignant human mammary ducts and acini to a much greater degree than in normal breast ducts. Immunohistochemical studies, however, have indicated that EMMPRIN is also present in normal breast ducts and some other epithelial structures (i.e. macrophages, endothelial cells, keratinocytes). The role of EMMPRIN in these non malignant cells remains to be determined. We have developed IgG monoclonal antibodies to human EMMPRIN which we have employed in immunohistochemistry, immunoassay, and Western blotting.

We have characterized the human EMMPRIN gene. The sum of the exon and intron sizes is 10.8 kb. Transcriptional factor consensus binding sequences have been identified.

We have transfected EMMPRIN cDNA into human MDA-MB-436 breast cancer cells and have demonstrated in 3 independent experiments that these cells are considerable more tumorigenic and invasive than plasmid transfected cancer cells. We have used green fluorescent protein as a convenient marker to examine for metastases in tumor-bearing mice. Intra abdominal metastases were noted with MDA-MB-436/EMMPRIN/GFP transfected cells; MDA-MB-436/GFP transfected cells were slow growing, did not metastasize locally, and did not succumb during the 12 weeks of observation.

Studies were performed to identify a binding site for EMMPRIN on fibroblasts; a putative binding protein of 58 kDa was identified using 2 different experimental approaches. Phage display experiments have demonstrated that EMMPRIN binds MMP-1 to the tumor cell surface in a similar fashion to the binding of progelatinase A to cell surfaces. An inducing effect of EMMPRIN on endothelial cell synthesis of MMPs was identified.

Based on these studies, we propose that inhibition of EMMPRIN function may provide a potential mechanism to alter the invasive process in breast cancer.

FIGURES

Figure 1: Immunohistochemical detection of EMMPRIN in human breast cancer tissue (top panel) employing mouse monoclonal antibodies to EMMPRIN. Note intense staining for EMMPRIN in tumor cells and weaker staining in surrounding fibroblasts. Hematoxylin and eosin staining of same tumor area (bottom panel).

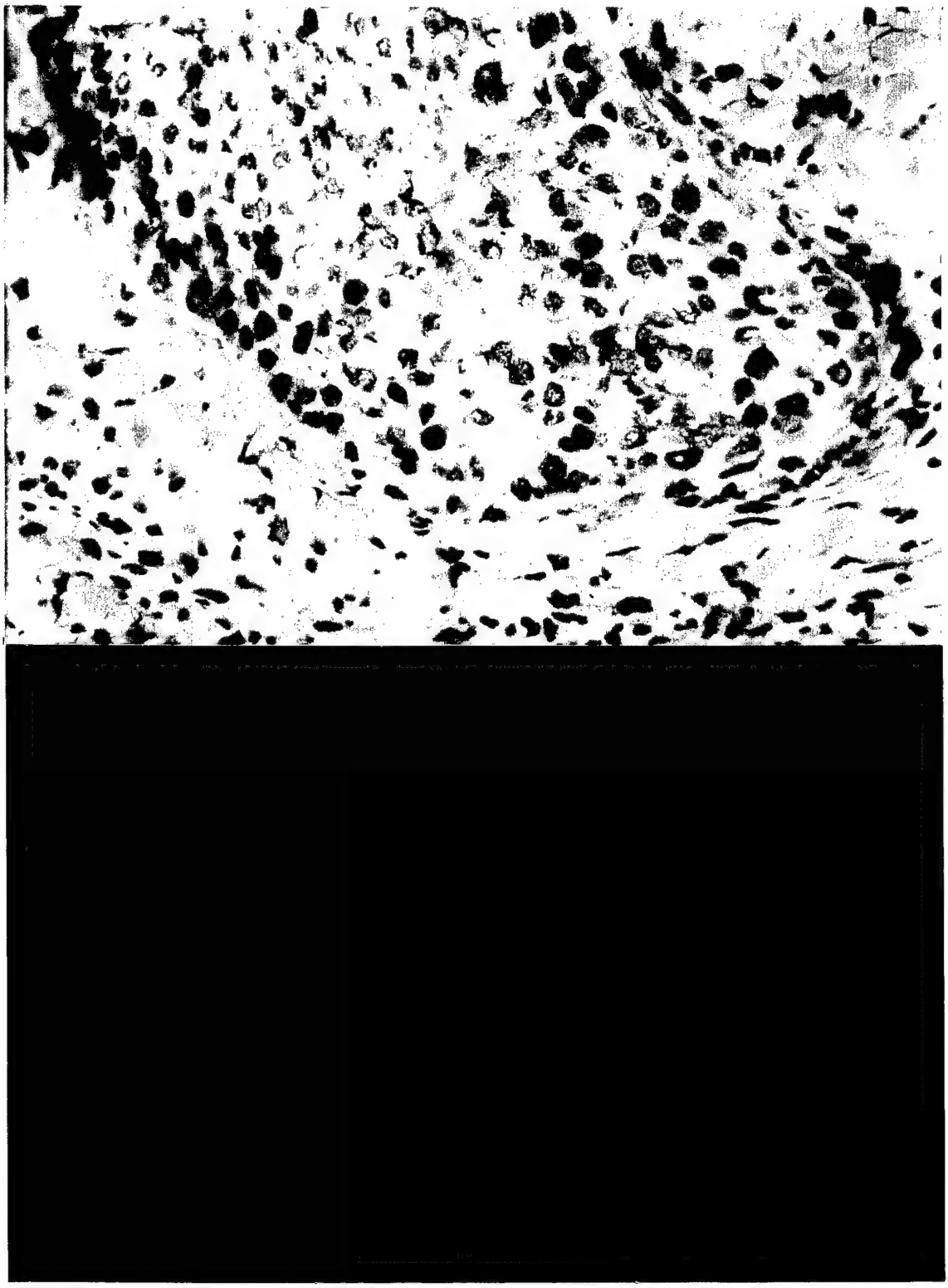
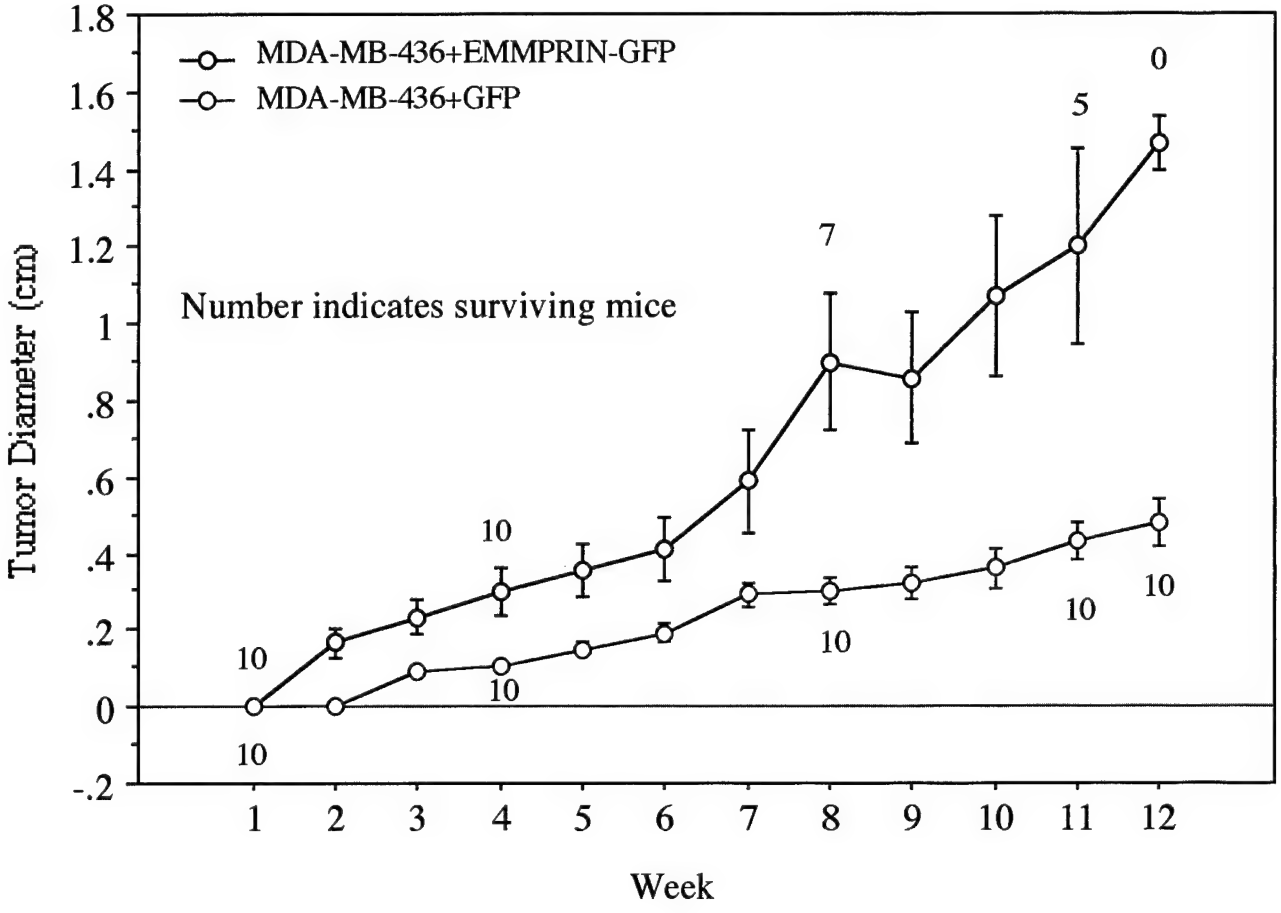


Figure 2: Transfection of EMMPRIN cDNA into human MDA-MB-436 breast cancer cells results in enhanced rate of tumor growth in vivo and early mortality after tumor cell implantation into mammary tissue of nude mice. The EMMPRIN transfected tumors are not only larger but more highly vascularized grossly (lower figure).



EMMPRIN-GFP GFP(alone)

Figure 3: EMMPRIN enhances secretion of interstitial collagenase, gelatinase A, and stromelysin-1 by human umbilical vein endothelial cells as demonstrated by Western blotting.

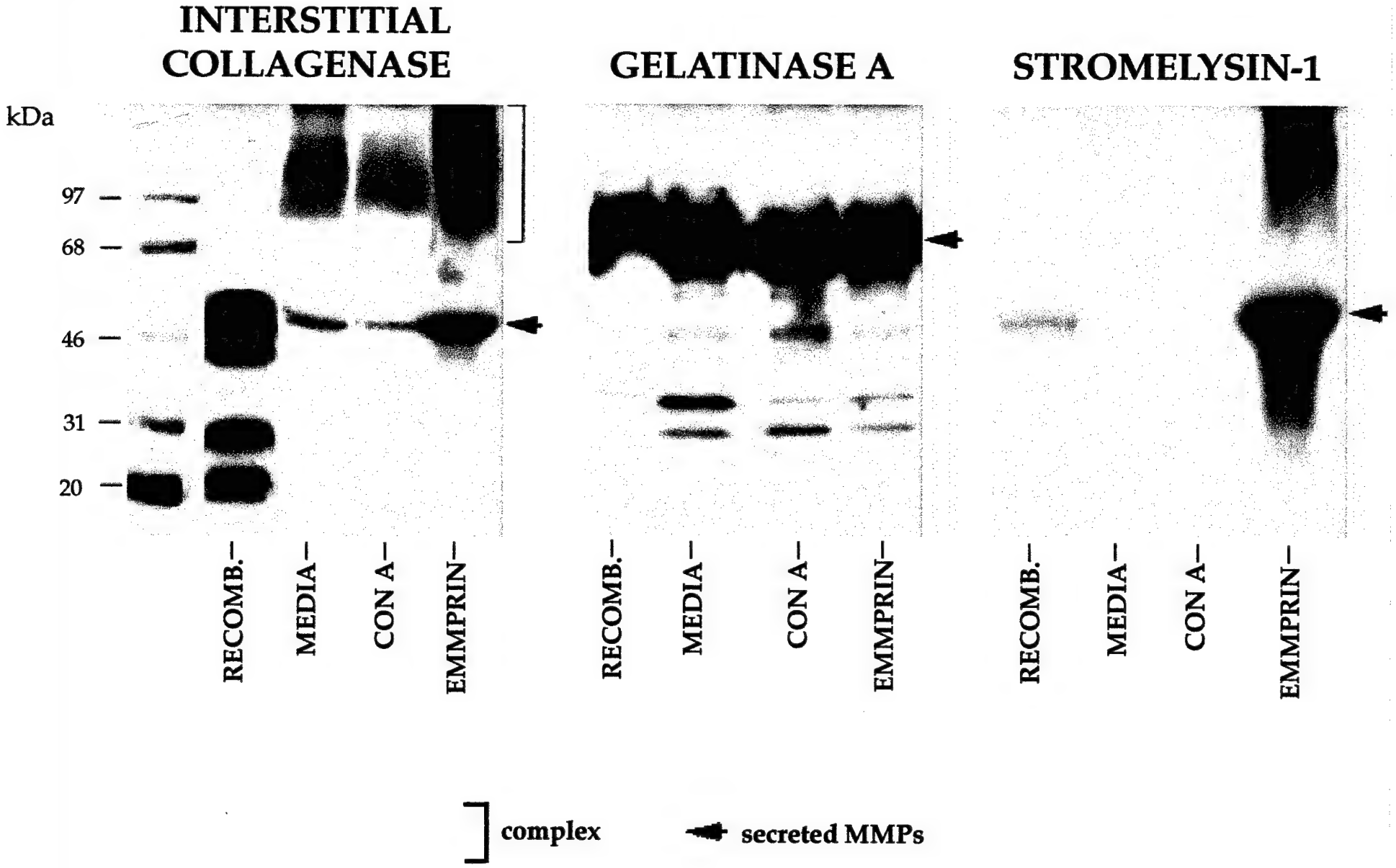
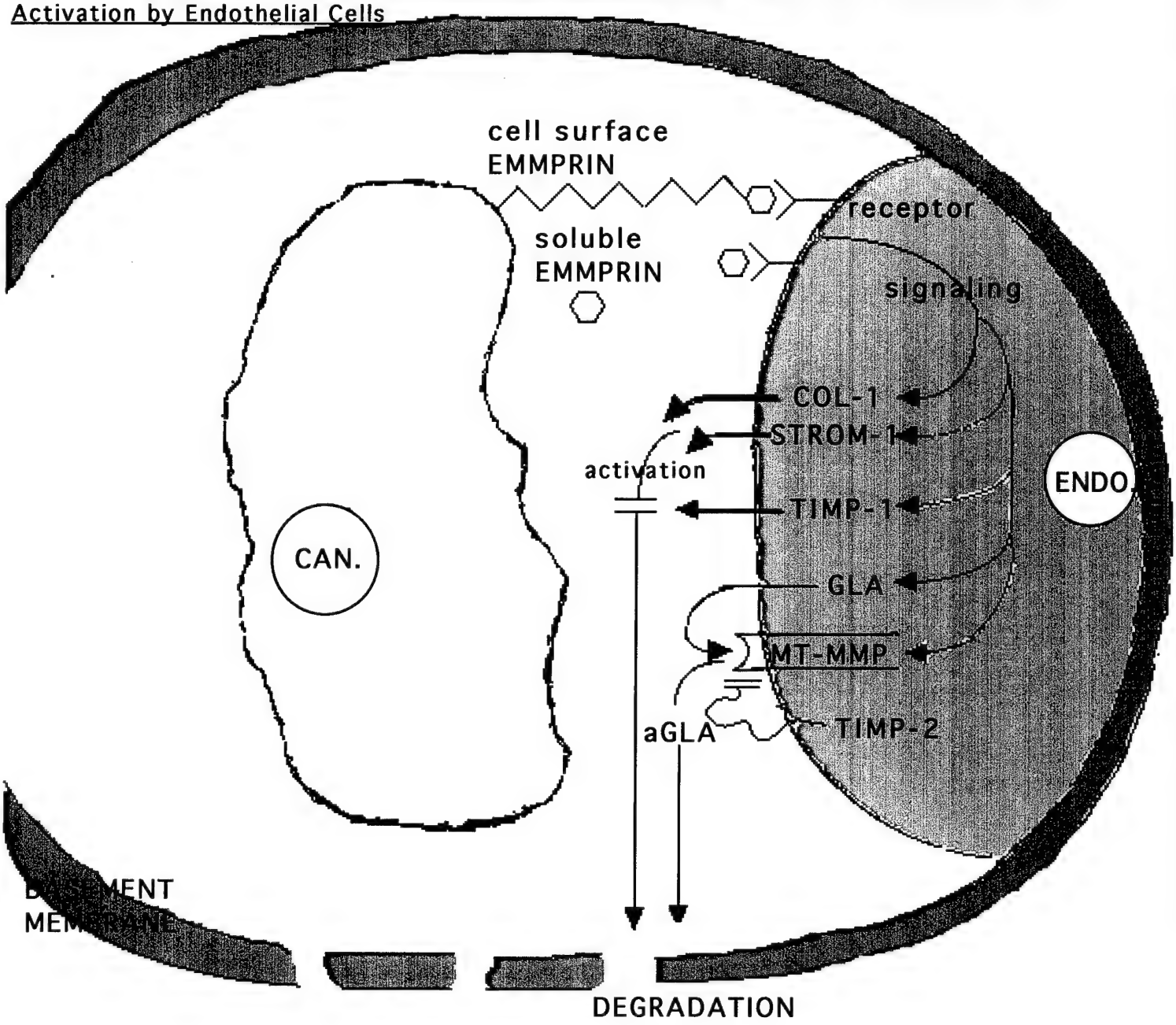


Figure 4: Hypothesis: Effect of Cancer Cell EMMPRIN on MMP and TIMP Production and Activation by Endothelial Cells



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Michelle Drews- Laboratory Technician
Cathleen Conner- Laboratory Technician
Hui Ming Guo- Post-Doctoral Fellow
Bryan P. Toole- Co-investigator

(11) APPENDICES
Reprints of recently published papers

[CANCER RESEARCH 55, 434–439, January 15, 1995]

The Human Tumor Cell-derived Collagenase Stimulatory Factor (Renamed EMMPRIN) Is a Member of the Immunoglobulin Superfamily[1]

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ABSTRACT

Tumor cell-derived collagenase stimulatory factor, renamed extracellular matrix metalloproteinase inducer (EMMPRIN), is a M_r ~58,000 glycoprotein which is located on the outer surface of human tumor cells and which interacts with fibroblasts to stimulate expression of several matrix metalloproteinases in the fibroblasts. In this study, we have used several approaches to isolate a complementary DNA encoding EMMPRIN. Several peptide sequences obtained from the isolated M_r 58,000 glycoprotein are found in the translated complementary DNA clone, verifying its identity. Computer database searches indicate that EMMPRIN is a member of the immunoglobulin superfamily and that the deduced amino acid sequence of EMMPRIN is identical to that recently reported for human basigin and M6 antigen, molecules of previously undetermined biological function.

INTRODUCTION

Degradation of extracellular matrix components of the basement membrane and interstitial matrix by MMPs[6] is a crucial step in tumor cell invasion and metastasis (1–3). The role of tumor cell-fibroblast interactions in regulation of MMP levels in neoplasms has been demonstrated by several investigators, including ourselves (4–7). The recent finding (8–11) that, *in vivo*, some tumor-associated MMPs are mainly synthesized in peritumoral fibroblasts, rather than in tumor cells themselves, is consistent with a major role for these interactions in tumorigenesis *in vivo*.

We have shown that tumor cells in culture stimulate fibroblasts to produce high levels of collagenase and that a factor (previously termed TCSF) that is associated with tumor cell membranes, but also released into medium conditioned by tumor cells, is responsible for this stimulation (6, 12, 13). We have immunoaffinity purified the M_r ~58,000 TCSF from a human lung carcinoma cell line, LX-1 (13, 14), and demonstrated that addition of this purified factor to cultured fibroblasts stimulates expression, not only of interstitial collagenase (MMP-1), but also of fibroblast-derived stromelysin-1 (MMP-3) and M_r 72,000 gelatinase (MMP-2) (15, 16). While immunohistochemical studies have shown that TCSF is highly enriched around the outer surface of tumor cells and absent from most normal cells *in vivo* (17), recent studies from our laboratory[7] have shown that TCSF is also present on the surface of normal human keratinocytes, where it presumably plays a role in regulating stromal MMPs (18). For these reasons, we have now renamed this factor EMMPRIN to indicate its role in extracellular matrix metalloproteinase induction via normal, as well as pathological, cellular interactions.

To help understand the chemical and biological nature of EMMPRIN and its relationship to other proteins, we have attempted to isolate cDNA clones for the protein. Oligonucleotide primers derived from peptide sequences were used to isolate EMMPRIN cDNAs by RT-PCR. Analysis of the cDNA-derived amino acid sequence of EMMPRIN indicates that it is a member of the immunoglobulin superfamily. Interestingly, the sequence is identical to two recently reported human cDNAs of unknown function, *i.e.*, human basigin (19) and M6 antigen (20). Thus, our studies provide one important function for these proteins, namely intercellular stimulation of MMP synthesis.

MATERIALS AND METHODS

Amino Acid Sequencing. Previously, we have reported the amino acid sequences for the NH_2-terminus of EMMPRIN and four peptides derived from EMMPRIN after trypsin digestion (14). We have now sequenced two more peptides derived from EMMPRIN in the same manner as described previously (14). Briefly, immunopurified EMMPRIN was subjected to SDS-PAGE and blotted to a nitrocellulose membrane. The EMMPRIN band was revealed by staining with Ponceau S. After destaining, the protein band was cut from the membrane and digested with trypsin at a ratio of 1:20 (w/w). The peptides were separated by reverse phase HPLC, and the samples were subjected to automated Edman degradation.

cDNA Synthesis. RNA was prepared from LX-1 cells by a routine procedure using guanidinium thiocyanate (21), and poly(A)$^+$RNA was isolated using the Mini Ribosep mRNA isolation kit according to the manufacturer's instructions (Collaborative Biomedical Products, Bedford, MA). First-strand cDNA was synthesized by reverse transcription of 1 μg of poly(A)$^+$ RNA using Moloney murine leukemia virus reverse transcriptase, according to the manufacturer's instructions (GIBCO-BRL, Gaithersburg, MD) in the presence of random hexamers or specific primers, depending on the desired reaction product. The resulting reaction mixture was digested with RNase H and used as a template for PCR.

DNA Amplification. PCR-amplified DNA fragments were generated with a Perkin-Elmer Cetus DNA thermal cycler (Norwalk, CT) using a gene amplification kit (Perkin Elmer) according to the manufacturer's instructions. Briefly, 100 μl of reaction mixture contained 100 ng of cDNA pool, 10 μl of 10X PCR buffer (provided in the kit), 16 μl of each deoxynucleotide triphosphate at 1.25 mM, 5 μl each of 20 μM primers, and 0.5 μl of Taq-DNA polymerase (2.5 units per assay). Samples were subjected to 30 cycles at the following conditions: 1 min at 94°C for denaturation; 1 min at 48°C for annealing; and 1.5 min at 72°C for elongation. A final elongation step consisted of a 72°C incubation for 10 min. Amplified products were separated by agarose gel electrophoresis and were identified by ethidium bromide staining. Where reamplification of a PCR product was necessary, samples were applied to low-melting point agarose gels (FMC, Rockland, ME); bands were excised, melted, and purified on Spin Bind extraction cartridges (FMC).

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[1] This work was supported by Grant CA 38817 (to C. B.) from the NIH.

[2] This article is dedicated to the memory of Dr. Chitra Biswas, who passed away August 26, 1993. Requests for reprints should be addressed to Dr. Bryan Toole at Department of Anatomy and Cellular Biology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111.

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[6] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; PCR, polymerase chain reaction, RT-PCR, reverse transcriptase-PCR; TCSF, tumor cell-derived collagenase stimulatory factor; bp, base pair(s); kb, kilobase.

[7] R. DeCastro, Y. Zhang, H. Guo, H. Kataoka, and C. Biswas. Human keratinocytes express EMMPRIN, an extracellular matrix metalloproteinase inducer, manuscript in preparation.

Generation of an Authentic cDNA for EMMPRIN Peptide #51. Two 17-mer, degenerate, oligonucleotide mixtures, based on the previously obtained amino acid sequence of EMMPRIN peptide #51 (14), were synthesized. One of these, termed A, was synthesized in the sense direction corresponding to amino acids 2–7; the other, termed C′, was synthesized in the antisense direction corresponding to amino acids 13–18. The sequences of the oligonucleotide mixtures are shown in Table 1. These mixtures were used as primers in RT-PCR to generate an authentic EMMPRIN cDNA corresponding to amino acid residues 2–18 of peptide #51, using LX-1 cell mRNA as the initial template. The primers had 8-bp adapters with restriction site sequences (*Eco*RI for the 5′-end of A and *Pst*I for the 5′-end of C′; not shown in Table 1), allowing subsequent cloning of the product in the event that difficulty was encountered in directly ligating the product into the pCRII vector. The resulting PCR products were sized by electrophoresis against known DNA markers in 6% agarose (NuSieve GTG low melting point agarose; FMC). Several products of different intensities were identified, including the expected 66-bp product corresponding to #51 (50-bp fragment plus 2 × 8 bp adapters at 5′ and 3′ ends). The band corresponding to this size was cut out from the agarose gel and reamplified using the same primers A and C′ as described above. After checking the size of the reamplified product, all of which was 66 bp, the reaction mixture was used directly for ligating into the pCRII vector system (Invitrogen). Recombinants were selected as white colonies on plates containing 5-bromo-4-chloro-3-indolyl-*b*-D-galactoside. Plasmid DNA was isolated from seven of these colonies, and the sizes of the inserts were analyzed by PCR amplification using M13 primers (forward and reverse), followed by agarose gel electrophoresis.

All seven recombinants had inserts of the expected sizes. DNA from two of these clones was sequenced by the dideoxy-mediated chain termination method (22) using a double-stranded DNA cycle sequencing kit (GIBCO-BRL). The nucleotide sequence of the inserts from both clones exactly matched the amino acid sequence of peptide #51 (Fig. 1). However, the nucleotide sequence varied at three positions, all within the primer sequences, as shown in *bold* in Fig. 1. This suggests that several of the degenerate primers were used by the template cDNA during amplification. The use of degenerate oligonucleotide mixtures as primers often leads to variations of this kind in the regions of the cDNA that correspond to the primers (23). To avoid these regions of variation, we designed primers (B/B′) based on the sequence of the central part of the cDNA (Fig. 1) for use in the overlap extension reactions described below.

Generation of cDNAs Corresponding to the 5′ and 3′ Regions of EMMPRIN. To obtain a cDNA that includes the region of EMMPRIN that is 5′ to peptide #51, primer B′ (designed as described above) was used in the PCR in combination with primer D, a degenerate mixture corresponding to part of the previously sequenced NH_2-terminal peptide #59, derived from EMMPRIN (Ref. 14, see Table 1). The PCR products were directly ligated into pCRII (Invitrogen) and used to transform *Escherichia coli*. Insert-containing white colonies were selected, and the plasmid DNAs were isolated, sized, and sequenced. One of these cDNAs, TALT5j, was used for further study.

To obtain a cDNA that includes the region of EMMPRIN that is 3′ to peptide #51, we used the rapid amplification of cDNA ends protocol as described previously (24). A pool of cDNA was prepared from poly(A)$^+$ RNA of LX-1 cells using the dT_{17} adapter primer E of 5′-GAATTCGAATTC-GATATCTTTTTTTTTTTTTTTT. The reaction mixture was then amplified in PCR using the dT_{17} adapter primer E and primer B, derived from peptide #51 (Table 1). After ligating the PCR products into the pCRII vector, transforming *E. coli*, and selecting white colonies, the plasmid DNA was isolated, and the inserts were sized and sequenced. One of these cDNAs, TALT3g, was used for further study.

Table 1 *EMMPRIN peptide sequences and derived oligonucleotide primers*

The amino acid sequences of the peptides for which corresponding oligonucleotide primers were synthesized are underlined. Oligonucleotide sequences A, B, and D are shown in the sense orientation, whereas B′ and C′ are in the antisense orientation. Primers A, C′, and D are degenerate mixtures; primers B and B′ are specific sequences derived as described in the text and Fig. 1. The nucleotide sequence, GARGA, highlighted in bold print at the 3′ end of primer D fortuitously matches a portion of the 5′ untranslated region of EMMPRIN (with the sequence, GAGGA) and thus amplified a cDNA beginning at this position (see Fig. 2).

Peptide sequence		Oligonucleotide primer[a]
#51 SELHIENLNMEADPGQYR	A:	5′-GAR-YTN-CAY-ATM-GAR-AA-3′
SELHIENLNMEADPGQYR	B:	5′-AAC-CTG-AAC-ATG-GAG-GCC-GA-3′
SELHIENLNMEADPGQYR	B′:	5′-TC-GGC-CTC-CAT-GTT-CAG-GTT-3′
SELHIENLNMEADPGQYR	C′:	5′-CZ-RTA-YTG-NCC-NGG-RTC-3′
#59 AAGTVFTTVEDLGSK	D:	5′-TTY-ACN-ACN-GTN-**GAR-GA**-3′

[a] M = A, C, or T; N = A, C, G, or T; Y = T or C; R = A or G; Z = G or T.

```
-------Primer A------>                          <-----Primer C'-------

GAA-CTT-CAC-ATT-GAG-AAC-CTG-AAC-ATG-GAG-GCC-GAT-CCC-GGC-CAA-TAC-CG

-E---L---H---I---E---N---L---N---M---E---A---D---P---G---Q---Y---R-
```

Fig. 1. Sequence of authentic cDNA for EMMPRIN peptide #51. The positions of primers A and C′ used to obtain the authentic cDNA for peptide #51 are shown on the *top line*; the nucleotide sequence of one of the cDNAs obtained from PCR is given on the *second line*; the amino acid sequence of peptide #51 (residues #2–18) is on the *third line*. The amino acid sequence deduced from the nucleotide sequence is identical to that obtained by amino acid sequencing. A second clone was sequenced, and differences were found in the positions highlighted in *bold print* at the third, sixth and forty-fifth positions of the nucleotide sequence; all of these lie within regions corresponding to the primers, and this variation was presumably due to the use of degenerate oligonucleotides as primers. Primers B (*underlined*) and B′ were designed from the central part of this cDNA.

Construction of a cDNA Encoding the Complete EMMPRIN Sequence. The inserts of the 5′ and 3′ cDNA clones for EMMPRIN (TALT5j and TALT3g) overlapped by 20 nucleotides and thus were used in the PCR-based, overlap extension technique (25) to yield a single cDNA with the complete open reading frame for EMMPRIN.

First, the cDNAs corresponding to the 5′ region (TALT5j) and the 3′ region (TALT3g) were amplified by PCR in two separate reactions. For TALT5j, a 30-mer sense primer, composed of a 13-bp *Bam*HI adapter at the 5′ end followed by a 17-mer corresponding to the 5′ terminus of TALT5j, was used (primer F, 5′-CGCGGATCCCGGCGAGGAATAGGAATCATG); the antisense primer was a 20-mer which corresponded to the 3′ terminus of TALT5j (primer B′ in Table 1). For TALT3g, a 20-mer sense primer corresponding to the 5′ terminus of TALT3g (primer B in Table 1) and the dT_{17} adapter primer E (5′-GAATTCGAATTCGATATCTTTTTTTTTTTTTTTT) were used. The amplified products were electrophoresed in agarose, and the bands corresponding to 0.6 kb (TALT5j) or 1.1 kb (TALT3g) were identified by ethidium bromide staining and excised.

In the third and final reaction, the gel-purified products from both the above reactions were used as templates for fusion by overlap extension (25). For this reaction, the 30-mer primer F (5′-most primer), used above for amplification of TALT5j, was used with the dT_{17} adapter primer E (3′-most primer). The 1.6-kb DNA product that was generated in this PCR was ethanol precipitated, agarose gel-purified, and subcloned into pBluescript. Sequencing of this cDNA confirmed its identity with the combined sequence of the two separate cDNAs. The complete sequence is shown in Fig. 2.

Northern Blot Analysis. Northern blot analysis was performed as described previously (16). Ten μg of total RNA was electrophoresed on a 1% agarose gel and transferred to nitrocellulose membrane. The blot was then hybridized overnight with EMMPRIN cDNA, which had been radiolabeled by nick-translation with ^{32}P-labeled dCTP. After washing, the filter was exposed to Kodak XR-5 film at −80°C for 48 h.

Deletion Analyses. The strategy used for making deletion constructs was adapted from that used to create the full-length EMMPRIN cDNA, *i.e.*, overlap extension (25). The primers used for the following reactions were primer F, 5′-CGCGGATCCCGGCGAGGAATAGGAATCATG-3′; primer G, 5′-ACG-GAGCCTCCGGGGTGAAGGCTGTGAAGTCG-3′; primer H, 5′-ACGGG-CCTCCCAGAAGCCACCTGGCCGCCCTC-3′; primer I, 5′-GCGTGCGC-AGCCACGAGAAGCGCCGGAAGCCC-3′; and primer J, 5′-TTCATCT-ACTAGTAGCGCCGGAA-3′.

The extracellular immunoglobulin domain I deletion construct was made as follows: (*a*) two PCRs, using EMMPRIN cDNA as template, were used to synthesize DNA fragments on each side of the desired deletion, one with the primers F (a *Bam*HI adapter plus the 5′ end of the EMMPRIN cDNA sequence) and reverse complement of G, and the other with primer G and the dT_{17} adapter primer E. Primer G is composed of sequences from each side of the desired deletion, *i.e.*, nucleotides 50–63 linked directly to 319–336 (Fig. 2). PCR products were analyzed and purified on 1% low-melting point agarose. DNA of expected sizes, 108 bp from the first reaction and 1306 bp from the second reaction, were cut from the gels and incubated in 300 μl H_2O at

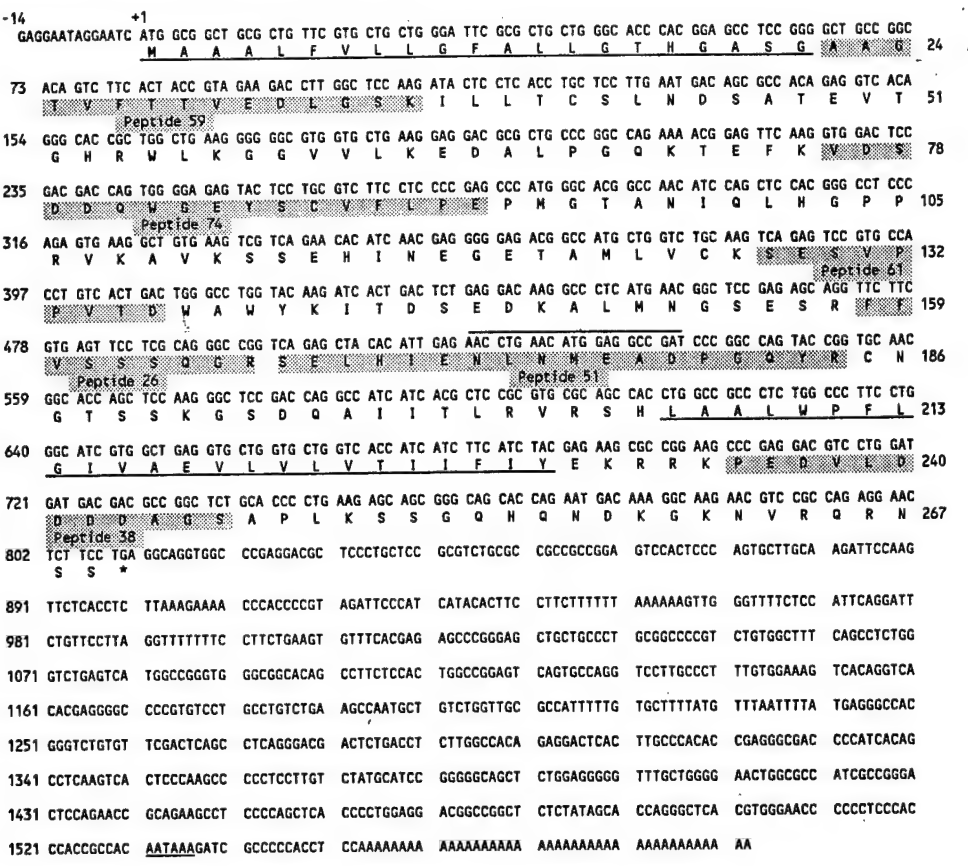

Fig. 2. Sequence of human EMMPRIN cDNA.[8] Nucleotide sequence and derived amino acid sequence for human EMMPRIN; nucleotides are numbered on the *left* and amino acids on the *right*. The putative signal and transmembrane amino acid sequences and the polyadenylation signal are *underlined*. The *shaded regions* indicate the deduced amino acid sequences that match the six peptides derived from EMMPRIN. The 20-nucleotide overlapping region of the 5′ and 3′ cDNAs, TALT5j and TALT3g, is indicated by a *line* above nucleotide positions #517–536. The stop codon is marked by an *asterisk*.

65°C for 10 min; (*b*) a PCR was performed with 10 ng each of these two fragments, plus primer F and the dT_{17} adapter. The PCR product was precipitated with 0.3 M ammonium acetate and 2 volumes of ethanol, *Eco*RI, and electrophoresed on 1% low-melting point agarose. DNA with the expected size, 1382 bp, was cut from the gel and purified on Spin bind extraction cartridge; and (*c*) the purified DNA was ligated into pBluescript, and used to transform XL-1 blue cells. Plasmid DNA was isolated from positive (white colony) transformants and confirmed by sequencing.

To create constructs with deletions in the extracellular immunoglobulin domain II, the transmembrane domain, and the cytoplasmic domain, the same approach was used with appropriate primer pairs. For deleting the extracellular immunoglobulin domain II, primers F and the reverse complement of H, and primer H and the dT_{17} adapter, were used to make fragments on each side of the desired deletion. Primer H contains sequences from both sides of the region we wished to delete, bases 305–318 linked directly to 610–627 (Fig. 2). To delete the transmembrane domain, the two fragments were made with primers F and the reverse complement of I, and primer I with the dT_{17} adaptor. Primer I consists of bases 602–615 linked to 688–705 (Fig. 2), sequences on each side of the desired deletion. Primer F and the dT_{17} adapter were used in the final PCR to make the fused deletion product from the two fragments. To delete the cytoplasmic domain, two premature stop codons were inserted into the EMMPRIN cDNA. PCR fragments were made from template cDNA using primers F and the reverse complement of J, and J with the dT_{17} adapter. Primer J corresponds to nucleotides 679–701 with T residues substituted for G and A at positions 688 and 691, respectively (Fig. 2) to create new termination codons. The two fragments were fused by overlap extension with primer F and the dT_{17} adapter. These deletion constructs were also subcloned into pBluescript and used to transform XL-1 blue cells.

To express each deletion construct, 20 μl of overnight cultures of each bacterial stock were added to 2 ml of Luria broth containing 0.2% glucose and 50 μg/ml ampicillin. After incubating at 37°C for 2 h, the cells were further incubated in the absence or presence of 0.5 mM isopropylthio-*b*-D-galactoside at 37°C for 3 h. The cells were collected by centrifugation and extracted by 30 μl of 2 × SDS-PAGE sample buffer. The cell extracts were separated on 15% SDS-PAGE and then analyzed for cross-reactivity with the monoclonal antibody E11F4 by Western blotting (26).

[8] The sequence reported in this publication has been deposited in the GenBank data base (accession no. L10240).

RESULTS

Amino Acid Sequences of Peptides Derived from EMMPRIN. Previously, we have reported the amino acid sequence of four peptides termed #26, #51, #59, and #61, obtained by HPLC fractionation of tryptic peptide digests of EMMPRIN (14). Subsequently, we have sequenced two additional peptides, #38 and #74, from EMMPRIN. The sequences of the six peptides are shown in Table 2.

Isolation of EMMPRIN cDNAs. Several attempts to obtain EMMPRIN cDNA clones by screening an LX-1 library with degenerate or best-guess oligonucleotide probes, derived from the peptide sequences, were unsuccessful. Therefore, the following steps were taken to obtain a cDNA for EMMPRIN. First, a small EMMPRIN-specific cDNA corresponding to a single peptide-derived sequence, peptide #51, was generated by RT-PCR with degenerate primers. Isolation of this cDNA confirmed the presence of an mRNA contain-

Table 2 *Amino acid sequences of peptides derived from EMMPRIN*

Peptides #38 and #74 (marked with an asterisk) are reported here for the first time; peptides #26, #51, #59, and #61 were reported previously (14).

Peptide #26:	Phe-Phe-Val-Ser-Ser-Ser-Gln-Gly-Arg
Peptide #38*:	Pro-Glu-Asp-Val-Leu-Asp-Asp-Asp-Asp-Ala-Gly-Ser-
Peptide #51:	Ser-Glu-Leu-His-Ile-Glu-Asn-Leu-Asn-Met-Glu-Ala-Asp-Pro-Gly-Gln-Tyr-Arg
Peptide #59:	Ala-Ala-Gly-Thr-Val-Phe-Thr-Thr-Val-Glu-Asp-Leu-Gly-Ser-Lys
Peptide #61:	Ser-Glu-Ser-Val-Pro-Pro-Val-Thr-Asp
Peptide #74*:	Val-Asp-Ser-Asp-Asp-Gln-Trp-Gly-Glu-Tyr-Ser-X-Val-Phe-Leu-Pro-Glu-

ing the EMMPRIN-derived sequence in LX-1 cells and provided us with a correct cDNA probe to be used for generation of larger EMMPRIN cDNAs.

After sequencing the small cDNA corresponding to peptide #51, a unique reverse complement primer derived from it was used in RT-PCR, together with a degenerate primer made from the amino terminal peptide #59, to obtain a longer cDNA. Sequencing of one of the isolated cDNAs (TALT5j; insert size, 0.6 kb) revealed the following characteristics: (*a*) as expected, the sequences at the 5′ and 3′ ends corresponded to the two primers, D and B′, which were based on portions of peptides #59 and #51, respectively; and (*b*) the complete sequences encoding peptides #26, #61, and #74 (Table 2) were present within the cDNA, demonstrating that the cDNA encodes an authentic EMMPRIN sequence.

An unexpected result from the above approach was that although the sequence of primer D was present at the 5′ end of the cDNA, the complete sequence corresponding to peptide #59, from which D is derived, was found to begin 72 nucleotide residues downstream from the primer sequence. Thus, it appears that primer D annealed with a region of the 5′ untranslated sequence of EMMPRIN and amplified a cDNA that begins in the 5′ untranslated region. A possible explanation of this event comes from comparison of the sequence of this cDNA with sequences recently obtained from an EMMPRIN cDNA derived from a human keratinocyte cDNA library.[7] The sequence at the 3′ end of primer D (Table 1) corresponds exactly with a sequence within the 5′ untranslated region, namely GAGGA, for the keratinocyte-derived as well as LX-1-derived EMMPRIN cDNAs. Therefore, this unexpected circumstance led to additional information about the 5′ untranslated end of our cDNA.

Thus, in summary, the 5′ cDNA (TALT5j) corresponds to nucleotide residues −14 to 536, defining +1 as A in the ATG start codon (Fig. 2). It begins with a portion of 5′ untranslated sequence, then contains a methionyl initiation codon at the start of a sequence encoding a region with the properties of a signal peptide (amino acid residues #1–21 in Fig. 2). This is followed by the sequence encoding peptide #59 (residues #22–36), corresponding to the NH_2-terminus of the mature protein. The cDNA continues through to the 3′ end primer, B′, encoding part of the amino acid sequence corresponding to peptide #51 (residues #173–178).

The rapid amplification of cDNA ends technique (24) was applied to obtain the 3′ cDNA, once more using a primer based on part of the authentic nucleotide sequence from the peptide #51 cDNA, together with a universal 3′ oligo dT_{17} adapter primer. Sequence analysis of the PCR products indicated that one of the inserts (TALT3g), 1.1 kb in size, begins at the 5′-terminus with the sequence corresponding to primer B, continues with the sequence of the COOH-terminus of peptide #51 (Table 1), and includes a perfect match with the amino acid sequence of peptide #38 (Table 2). The sequence of TALT3g corresponds to nucleotides 517–1592 in Fig. 2. This sequence also contains a stop codon at nucleotide positions #808–810 and a 3′ untranslated region containing a poly-adenylation signal and a poly(A) tail (Fig. 2).

Finally, the 5′ and 3′ cDNAs generated above were fused by the overlap extension method (25) to yield a single 1.6-kb cDNA. The full sequence of this cDNA, its deduced translation product, and the tryptic peptide sequences are shown in Fig. 2.

Northern Blot of mRNA for EMMPRIN. To ascertain the size of mRNA for EMMPRIN, total RNA derived from LX-1 cells was analyzed by Northern blotting using the 5′ cDNA (TALT5j) as a probe. A single band of ~1.7 kb was observed (Fig. 3). This mRNA size is close to the size of cDNA obtained after fusion of the TALT5j and TALT3g cDNAs, *i.e.*, 1.6 kb.

Fig. 3. Northern blot of LX-1 RNA probed with EMMPRIN cDNA. Ten μg of total RNA was loaded and probed with nick-translated, radiolabeled cDNA (TALT5j). Total radioactivity used was 1×10^6 cpm/ml, and the duration of film exposure was 18 h.

Analysis of EMMPRIN cDNA Sequences. The Northern blots indicate that the EMMPRIN composite cDNA corresponds to all but about 100 nucleotides of the mRNA. Since the clone contains a poly(A) tail, this means that the 100 bases are probably located at the 5′-end of the 5′ untranslated region, making the total untranslated region about 115 nucleotides in length.

The cDNA encodes a 269-amino acid residue polypeptide that contains a putative signal peptide of 21 amino acid residues, an extracellular domain of 185 amino acid residues, a putative transmembrane region (residues 206–229), and a carboxy-terminal cytoplasmic domain of 39 amino acid residues (230–269). The transmembrane region includes three leucines (residues 206, 213, and 220) and a phenylalanine (residue 227), occurring every seventh residue, a characteristic feature of the leucine zipper motif (Fig. 2). The extracellular region contains four cysteinyl residues spaced in a manner that gives rise to two distinct domains with the characteristics of proteins in the immunoglobulin superfamily. These residues in EMMPRIN are located at amino acid residue positions 41, 87, 126, and 185.

Expression of Recombinant EMMPRIN Protein and Deletion Constructs. Although the EMMPRIN cDNA encodes amino acid sequences identical to those of peptides directly isolated from immunoaffinity-purified EMMPRIN protein, we reconfirmed the identity of the EMMPRIN cDNA by showing that the recombinant protein is recognized by activity-blocking monoclonal antibody. To accomplish this, the recombinant protein was expressed in pBluescript and then assayed by Western blotting with EIIF4, a monoclonal antibody that blocks the activity of EMMPRIN protein from LX-1 cells (13, 14). As shown in Fig. 4*B*, *Lane 2*, EIIF4 reacts with the recombinant EMMPRIN protein. Two immunoreactive bands were obtained; these correspond in size to the two forms of EMMPRIN noted previously (14) and most likely arise by proteolysis.

The bacterial recombinant protein was also used to determine the approximate location of the epitope for E11F4, taking advantage of the lack of posttranslational modifications that would interfere with such studies on the native, immunoaffinity-purified protein. Modified EMMPRIN expression plasmids were made containing deletions in four locations. As seen in Fig. 4*A*, these deletions were: (*a*) deletion of the extracellular immunoglobulin domain I (ΔECI); (*b*) deletion of the extracellular immunoglobulin domain II (ΔECII); (*c*) deletion of the transmembrane domain (ΔTM); and (*d*) deletion of the cytoplasmic domain (ΔCYT). XL-1 blue cells were transformed with the deletion expression pBluescript plasmids, and the expressed proteins were analyzed by SDS-PAGE and Western blotting with the monoclonal antibody E11F4. As seen in Fig. 4*B*, all the plasmids produce protein that is immunoreactive, except for the plasmid lacking immunoglobulin domain I.

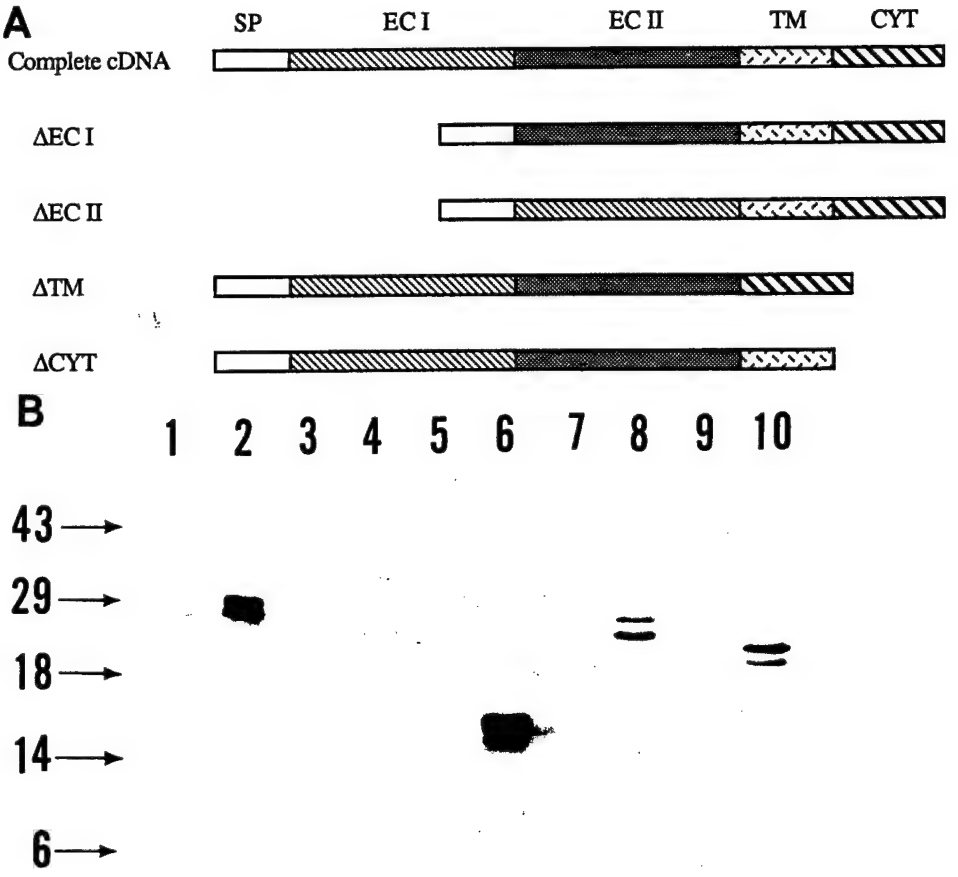

Fig. 4. Localization of EIIF4 epitope in recombinant EMMPRIN. *A*, strategy for deleting the four major domains of the EMMPRIN cDNA. ΔECI and ΔECII, deletion of one of two immunoglobulin-like domains within the extracellular region; ΔTM, deletion of transmembrane domain; ΔCYT, deletion of cytoplasmic domain; *SP*, signal peptide; *B*, Western blot. The transformed XL-1 blue cells were incubated in the absence (*Lanes 1, 3, 5, 7,* and *9*) or presence (*Lanes 2, 4, 6, 8,* and *10*) of 0.5 mM isopropyl-β-D-thiogalactopyranoside and processed for Western blotting. *Lanes 1* and *2*, recombinant EMMPRIN without deletion; *Lanes 3* and *4*, ΔECI; *Lanes 5* and *6*, ΔECII; *Lanes 7* and *8*, ΔTM; *Lanes 9* and *10*, ΔCYT. Molecular weight marker positions in kilodaltons are shown.

These results demonstrate that our cDNA encodes the protein which is reactive with our activity-blocking monoclonal antibody and that the antibody epitope exists in the extracellular immunoglobulin domain I. This, in turn, implies that the functional site of the metalloproteinase stimulatory activity of EMMPRIN is likely to be localized to sequences contained in the immunoglobulin domain I region.

DISCUSSION

We have isolated and fused two overlapping cDNA clones, using the polymerase chain reaction, which together encode the complete, 269-amino acid open reading frame for EMMPRIN. The identity of the clones was confirmed by comparison to several peptide sequences derived from immunoaffinity-purified EMMPRIN. Recognition of the translation product by the activity-blocking monoclonal antibody EIIF4 further confirms the identity of the cDNAs as the desired EMMPRIN clones. In addition, we have recently isolated an EMMPRIN cDNA from a human keratinocyte cDNA library. The open reading frame of the latter cDNA has an identical sequence to the cDNA obtained in the present study by PCR-based techniques, except for two nucleotide residues; however, the deduced amino acid sequences are identical for the two cDNAs.[7] Finally, we have recently demonstrated that recombinant EMMPRIN isolated from CHO cells transfected with EMMPRIN cDNA stimulates metalloproteinase production in fibroblasts.[9]

The composite cDNA obtained in the present study has a small 5′ untranslated region, followed by an initiation codon and sequences that have the properties of a signal peptide sequence, when using the rules of von Heijne (27). The subsequent codons agree perfectly with our amino terminal peptide sequence for the mature protein, demonstrating that the signal peptide sequence is genuine. The 248 codons after the signal sequence encode a 185-amino acid extracellular domain consisting of two regions characteristic of the immunoglobulin superfamily, followed by 24-amino acid residues comprising the transmembrane domain and a 39-amino acid cytoplasmic domain. The 248-amino acid residues of the mature protein correspond to an approximate molecular weight of 27,000. However, the purified protein has a larger molecular weight of ~58,000 (13). This difference is mainly due to glycosylation of the protein[7] (20).

We have shown previously that EMMPRIN is present at the surface of tumor cells (13) and has the properties of a membrane-intercalated protein (12). On the basis of these findings, we proposed previously that EMMPRIN is attached to the plasma membrane via a transmembrane domain and interacts with a receptor on fibroblasts via an extracellular domain (1). The presence in the cDNA of sequences typical of a signal peptide and a transmembrane region is consistent with EMMPRIN being an integral plasma membrane protein.

After the termination codon, the cDNA contains a 3′ untranslated region ending in a poly(A) tail. Northern blot analysis indicates that the mRNA for EMMPRIN is ~1.7 kb in size, which is approximately the same as that of the fused EMMPRIN cDNA. It is evident, however, that a portion of the 5′ untranslated region is lacking from this cDNA.

The EMMPRIN cDNA sequences were used in computer searches of the EMBL and GenBank data bases to detect homology with other known proteins. These searches revealed that the EMMPRIN cDNA is identical to two other human cDNAs, encoding proteins of un-

[9] H. Guo, M. Gordon, B. P. Toole, and C. Biswas, Recombinant human tumor cell EMMPRIN stimulates fibroblast metalloproteinase production, manuscript in preparation.

known function, basigin (19) and the M6 antigen (20). Therefore, our studies, which have been performed from a functional standpoint over the course of many years (4, 6, 12–16), have elucidated at least one biological function of these molecules. We are changing our former designation of the molecule from TCSF to EMMPRIN and would suggest that all the above proteins now be designated EMMPRIN, because the acronym more accurately implies at least one definitive function of the glycoprotein, *i.e.*, stimulation of MMP synthesis via cell-cell interaction.

The fact that EMMPRIN is a member of the immunoglobulin superfamily is also compatible with the idea that, in similar fashion to the N-CAM, I-CAM, and other related subgroups of the immunoglobulin superfamily (28), it acts via cell-cell interactions (1). We are currently attempting to identify the molecule on the surface of fibroblasts that interacts with tumor cell-derived EMMPRIN, causing increased fibroblast MMP production. Our recent finding that EMMPRIN is expressed in keratinocytes and localized in the basal layers of the epidermis[7] suggests the possibility that EMMPRIN may have a natural function in embryonic development or wound healing by causing dermal fibroblasts to increase their MMP production, thus facilitating tissue remodeling (18). The antibody to M6 antigen localizes EMMPRIN to granulocytes in patients with rheumatoid arthritis (20), possibly indicating a role for EMMPRIN in stromal MMP production and the consequent matrix degradation that occurs in the arthritic joint. Thus, we propose that EMMPRIN and related molecules are important mediators of matrix remodeling in normal and pathological tissues.

With respect to tumorigenesis, it has become clear that: (*a*) MMPs are crucial to the process of tumor cell invasion through basement membranes and interstitial matrices (1–3); and (*b*) in the case of interstitial collagenase, stromelysin, and M_r 72,000 gelatinase (type IV collagenase), the MMPs involved are produced mainly by peritumoral fibroblasts rather than by the tumor cells themselves (8–11). Since tumor cell-derived EMMPRIN causes a significant increase in the levels of these three enzymes in human fibroblasts (6, 13–16) and since EMMPRIN is associated with the surface of many types of tumor cells *in vivo* and *in vitro* (13, 17),[10] it is very likely that EMMPRIN is a central factor in the stimulation of MMPs required for tumor invasion and metastasis.

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ARTICLE

Tumor Collagenase Stimulatory Factor (TCSF) Expression and Localization in Human Lung and Breast Cancers

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SUMMARY Tumor cell-derived collagenase stimulatory factor (TCSF) stimulates in vitro the biosynthesis of various matrix metalloproteinases involved in tumor invasion, such as interstitial collagenase, gelatinase A, and stromelysin 1. The expression of TCSF mRNAs was studied in vivo, using in situ hybridization and Northern blotting analysis, in seven normal tissues and in 22 squamous cell carcinomas of the lung, and in seven benign proliferations and in 22 ductal carcinomas of the mammary gland. By in situ hybridization, TCSF mRNAs were detected in 40 of 44 carcinomas, in pre-invasive and invasive cancer cells of both lung and breast cancers. TCSF mRNAs and gelatinase A mRNAs were both visualized in the same areas in serial sections in breast cancers, and were expressed by different cells, tumor cells, and fibroblasts. The histological results were confirmed by Northern blot analysis, which showed a higher expression of TCSF mRNAs in cancers than in benign and normal tissues. These observations support the hypothesis that TCSF is an important factor in lung and breast tumor progression. **(J Histochem Cytochem 45:703–709, 1997)**

KEY WORDS
TCSF
metalloproteinases
tumor invasion

TUMOR INVASION is a multistep process that involves the degradation of basement membrane and interstitial matrix components by proteolytic enzymes. Many data actually support an important role for the matrix metalloproteinases (MMPs) in this proteolytic event. High levels of MMPs have been described in many cancer cell lines that display high invasive capacity (Gilles et al. 1994; Monsky et al. 1994; Taniguchi et al. 1992,1994; Bernhard et al. 1990; Bonfil et al. 1989). Such an observation has been recently extended to a newly discovered member of the MMPs, membrane type matrix metalloproteinase 1 (MT-MMP-1), which has also been shown to be correlated with in vitro invasiveness (Sato et al. 1994; Okada et al. 1995; Gilles et al. 1996). In vivo, MMPs have also been associated with the metastatic progression of many human cancers (Davies et al. 1993; Clavel et al. 1992; Levy et al. 1991; Monteagudo et al. 1990). However, recent in vivo data obtained by in situ hybridization (ISH) have shown that interstitial collagenase, gelatinase A, stromelysins, and MT-MMP-1 are mostly synthesized by fibroblasts localized near tumor cell clusters (Okada et al. 1995; Pyke et al. 1993; Poulsom et al. 1992,1993; Polette et al. 1991,1993,1996; Basset et al. 1990).

The specific detection of MMPs in peritumoral fibroblasts has led to the hypothesis that tumor cells might induce the synthesis of these enzymes implicated in cancer dissemination. In agreement with such an idea, several investigators have demonstrated cooperation between tumor cells and fibroblasts in vitro in the regulation of several MMPs, such as interstitial collagenase (Noël et al. 1993; Hernandez et al. 1985; Biswas 1984,1985; Bauer et al. 1979) and gelatinase A (Ito et al. 1995; Noël et al. 1994). Furthermore, a tumor cell-derived collagenase stimulatory factor (TCSF) also present in tumor cell-conditioned media, was isolated and purified from the plasma membranes of a human lung carcinoma cell line (Ellis et al. 1989). This factor is a glycoprotein of 58 kD and was recently

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identified as a member of the immunoglobulin superfamily (Biswas et al. 1995). In addition to enhancing interstitial collagenase synthesis (Nabeshima et al. 1991), purified TCSF stimulates gelatinase A and stromelysin 1 expression by fibroblasts (Kataoka et al. 1993). Immunohistochemical studies employing a monoclonal antibody directed against TCSF have shown that TCSF is localized to the outer surface of cultured lung cancer cell lines (Ellis et al. 1989). The same distribution was seen in tumors of urinary bladder, in which TCSF was detected at the periphery of cancer cells but not in surrounding stromal cells (Muraoka et al. 1993). Furthermore, TCSF was also localized in tumor cells by immunohistochemistry in invasive and in situ ductal breast cancers (Zucker and Biswas 1994).

On the basis of limited information concerning TCSF localization in cancer tissue, the role of TCSF in tumor progression remains unclear. In the present study, to clarify the cell origin of TCSF and to study its role in cancer invasion, we performed in situ hybridization and Northern blot analysis on human lung and breast carcinomas as well as on normal tissues.

Materials and Methods

Source of Tissue

The tissue was obtained from 22 lungs resected for squamous cell carcinomas of Stages I (10 cases), II (eight cases), and III (four cases) according to the TMN classification, from seven normal lung samples, from 22 ductal breast cancers of Grade 1 (four cases), Grade 2 (14 cases), and Grade 3 (four cases) according to the Scarf and Bloom classification, and from seven benign breast proliferations (two fibrocystic disease and five fibroadenoma).

Tissue Preparation

Part of the samples were frozen in liquid nitrogen for Northern blot analysis and the remainder were fixed in formalin and embedded in paraffin for in situ hybridization.

In Situ Hybridization Localization

Tissue sections (5 μm) were deparaffinized, rehydrated, and treated with 0.2 M HCl for 20 min at room temperature, followed by 15 min in 1 μg/ml proteinase K (Sigma Chemical; St Louis, MO) in Tris–EDTA–NaCl, 37C, to remove basic proteins. The sections were washed in 2 × SSC (sodium saline citrate), acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, and hybridized overnight with ^{35}S-labeled (50C) anti-sense RNA transcripts. TCSF cDNA (1700 BP) and gelatinase A (1500 BP) (a gift from G. Murphy; Cambridge, UK) were subcloned into pBluescript II SK+/− plasmid and pSP64, respectively, and used to prepare ^{35}S-labeled RNA probes. Hybridizations were followed by RNAse treatment (20 μg/ml, 1 h, 37C) to remove unhybridized probe and two stringent washes (50% formamide–2 × SSC, 2 hr at 60C) before autoradiography using D 19 emulsion (Kodak; Rochester, NY). Slides were exposed for 15 days before development. The controls were performed under the same conditions, using ^{35}S-labeled sense RNA probes. All slides were counterstained with HPS (hematoxylin–phloxin–safran), mounted, and examined under a Zeiss Axiophot microscope.

In Situ Hybridization Quantitation by Image Cytometry

Quantitation of the number of hybridization grains/μm^2 was performed with the help of an automated image analyzer, the DISCOVERY system (Becton–Dickinson; Mountain View, CA). After thresholding, the number of grains are counted automatically on at least six fields at high magnification (× 500). At this magnification, one field measures 12,688 μm^2. We performed these measurements on six different samples (three lung and three breast carcinomas) in which we found normal, in situ, and invasive areas on the same tissue section. Statistical analyses of TCSF mRNA expression levels were compared using the non-parametric Mann–Whitney *U*-test. Data were expressed as mean of dots/μm^2 ± SEM. *p* values equal to or less than 0.05 were considered significant.

Northern Blot Analysis

Extraction of total RNA from tissues was performed by RNAzol treatment (Biogenesis; Bournemouth, UK). Ten μg of each RNA was analyzed by electrophoresis in 1% agarose gels containing 10% formaldehyde and transferred onto nylon membranes (Hybond-N; Amersham, Poole, UK). The membrane was hybridized with the cDNA probe encoding TCSF (1700 BP) labeled with ^{32}P using random priming synthesis (5 × 108 cpm/μg) (Dupont de Nemours; Bruxelles, Belgium). The filters were exposed for 1 day. Membranes were rehybridized to a ubiquitous 36B4 gene probe, which served as a control. Signal intensities were recorded using a CD 60 Desaga (Heidelberg, Germany) laser-scanning densitometer and TCSF levels (in arbitrary units) were standardized with their corresponding 36B4 levels to obtain values independent of RNA quantities deposed onto gels. Statistical analyses of TCSF expression levels were compared using the non-parametric Mann–Whitney *U*-test. Data were expressed as mean ± SEM. Differences or similarities between two populations were considered significant when confidence intervals were <95% ($p<0.05$).

Results

Lung Lesions

By Northern blotting, TCSF transcripts were detected in 18 of 22 carcinomas. Quantitative analysis showed significantly higher ($p<0.05$) TCSF mRNA expression in lung carcinomas than in peritumoral lung tissues (Figures 1 and 2A). However, no significant differences between the TCSF mRNA levels were found in accordance with the TNM stage (Figure 2A).

With in situ hybridization, pre-invasive and invasive cancer cells were labeled in 18 of 22 tumors examined (the same positive samples as those found by

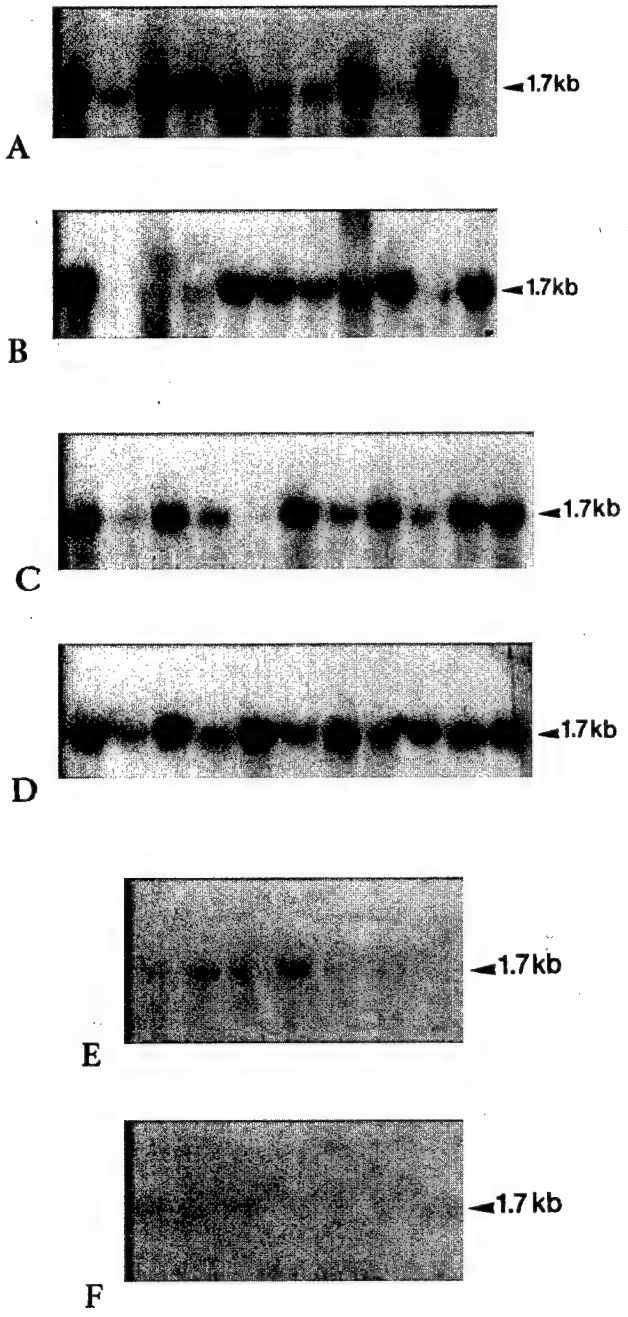

Figure 1 Northern blotting analysis of total RNA extracted from 22 lung carcinomas (**A,B**), 22 breast carcinomas (**C,D**), seven peritumoral normal lung tissue samples (**E**), and seven benign breast proliferations (**F**). A TCSF transcript of 1.7 KB is detected in tumor samples (40/44), whereas it is very weak or undetectable in normal tissue or benign proliferations.

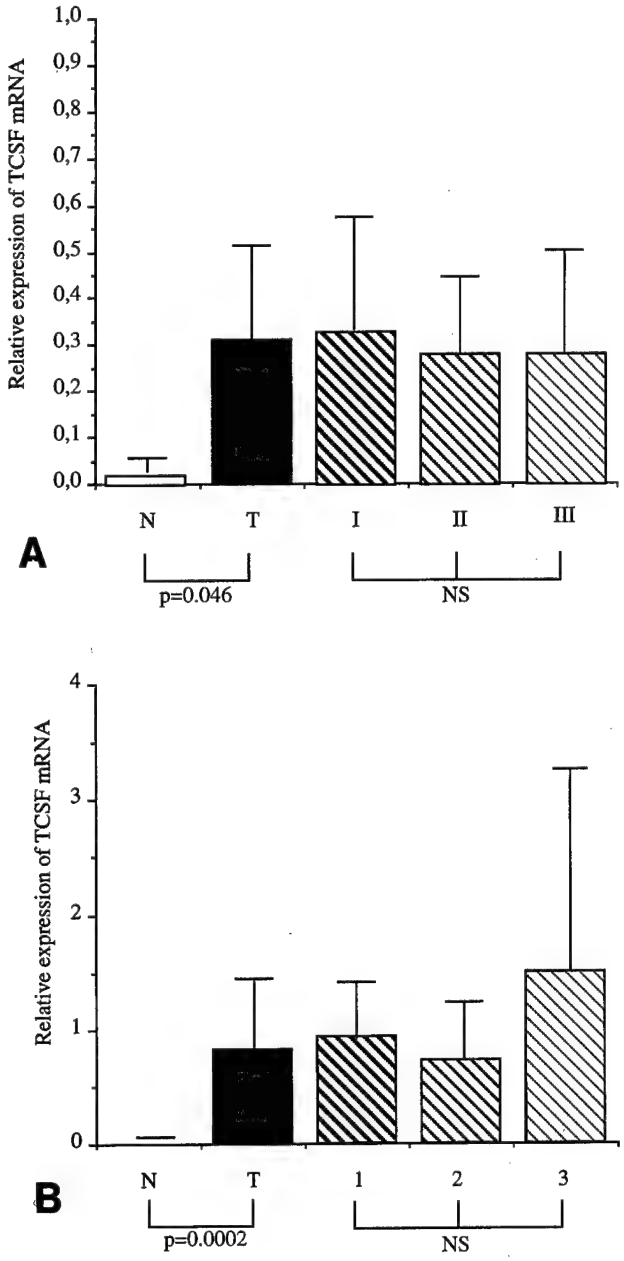

Figure 2 Comparison of TCSF mRNA levels (arbitrary units) according to tissue pathology and to the TNM stage and Scarf and Bloom grading in lung (**A**) and breast (**B**) samples. (**A**) Lung tumor samples (T) expressed significant higher TCSF mRNA levels than non-tumor samples (N). However, no significant differences were found according to the TNM stage of lung carcinomas. (**B**) Breast tumor samples (T) expressed high TCSF mRNA levels whereas no signal was detected in benign breast tissues (N). Statistical analysis did not find any significant differences according to the Scarf and Bloom grade of breast carcinomas.

Northern blot analysis). Stromal cells surrounding labeled invasive cancer cells, were always negative (Figure 3A). Normal (Figure 3C) or squamous metaplastic epithelium and bronchial glands did not express any TCSF transcripts. Moreover, in the normal or emphysematous adjacent lung, pulmonary alveolar macrophages identified by the CD68 monoclonal antibody (Dako; Carpinteria, CA) on serial sections (not shown) were particularly rich in TCSF mRNAs (Figure 3D). In the three cases analyzed by image cytometry, TCSF mRNAs were significantly expressed in tu-

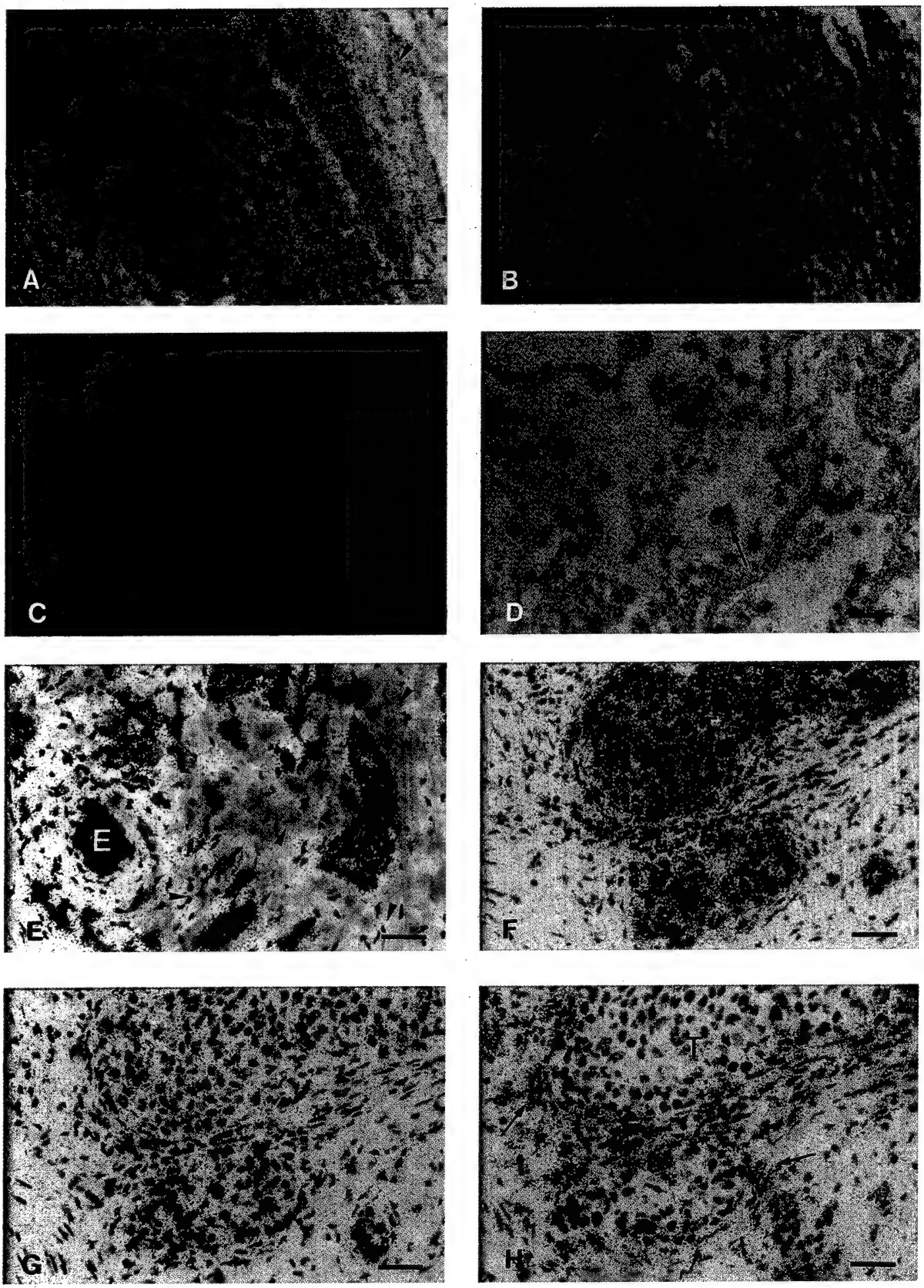

Figure 3 Localization of TCSF in lung cancers and breast cancers. **(A)** TCSF mRNAs are detected in invasive cancer cells (T) in lung carcinoma using an anti-sense probe, whereas stromal cells (arrowheads) are negative. Bar = 70 μm. **(B)** Same area treated with TCSF sense RNA probe. Bar = 70 μm. **(C)** Epithelial cells (E) of normal lung tissue do not express TCSF mRNA. (Bar = 70 μm). **(D)** TCSF mRNA is also present in many alveolar macrophages (arrow). Bar = 70 μm). **(E)** Intraductal breast tumor cells (T) express TCSF mRNAs, whereas epithelial adjacent cells (E) and stromal cells (arrowheads) do not show any hybridization grains. Bar = 70 μm. **(F)** Invasive tumor cells (T) express TCSF mRNAs, whereas stromal cells are negative. Bar = 70 μm. **(G)** Same area treated with TCSF sense RNA probe. Bar = 70 μm. **(H)** Gelatinase A mRNAs are localized in fibroblasts (arrow) in close contact to tumor clusters (T) in serial sections of breast carcinoma. Bar = 70 μm.

mor cell nests of both pre-invasive (1.68 ± 0.22 /μm^2) and invasive areas (2.14 ± 0.35 /μm^2) compared to the extracellular control compartment (0.19 ± 0.02 / μm^2) and normal tissue (0.23 ± 0.04 /μm^2) ($p<0.05$).

Breast Lesions

By Northern blotting, TCSF mRNAs were detected in the 22 breast carcinomas. Quantitative analysis showed significantly higher ($p<0.05$) expression of TCSF mRNAs in breast carcinomas than in benign breast lesions (Figures 1 and 2B). No significant differences between the TCSF mRNA levels were found in accordance with the Scarf and Bloom staging (Figure 2B).

With in situ hybridization, benign proliferations and normal mammary areas mixed with cancer cells or adjacent to cancer areas did not show any hybridization grains (Figure 3E), whereas the TCSF mRNAs were detected in cancer cells in pre-invasive and invasive areas (Figure 3F) of all 22 tumors examined. Stromal cells did not contain any hybridization grains. In the three cases studied by quantification, the density of markers in both pre-invasive ($2.14 \pm 0.48/\mu m^2$) and invasive ($2.95 \pm 0.92/\mu m^2$) areas was significantly higher than in the extracellular control compartment ($0.23 \pm 0.12/\mu m^2$) and the normal areas ($0.28 \pm 0.10/\mu m^2$) ($p<0.05$). On serial sections, TCSF mRNAs were localized in cancer cells, whereas fibroblasts close to tumor clusters expressed mRNAs encoding gelatinase A (Figures 3F and 3H) in the same areas.

Discussion

In this study we clearly showed the presence of mRNA encoding TCSF in epithelial tumor cells of lung and breast carcinomas. In agreement with our observations, previous immunohistochemical studies detected TCSF in cancer cells in breast (Zucker and Biswas 1994) and in bladder carcinoma (Muraoka et al. 1993). In the latter study, no staining was found in epithelial cells in non-neoplastic urothelium, except in superficial umbrella cells. However, Zucker and Biswas (1994) reported that TCSF is also present in normal breast ductules and lobules near in situ carcinoma areas, describing a more extensive distribution of TCSF compared with our data obtained by in situ hybridization (ISH). No TCSF transcripts were detected in normal epithelial cells in both non-neoplastic breast and lung lesions. It is therefore likely that normal epithelial cells express low levels of the TCSF mRNAs that we could not detect by ISH but that could produce amounts of the TCSF proteins detectable by immunohistochemistry. The presence of TCSF mRNAs in benign and normal tissues was confirmed by our Northern blot analysis, identifying weak expression of TCSF mRNAs in those samples. Furthermore, our Northern blot analysis strengthened our ISH data because a higher significant abundance of TCSF transcripts was found in both breast and lung carcinomas than in benign and normal samples. The detection of TCSF mRNAs in intraepithelial cancer areas of the lung and mammary gland indicates that TCSF mRNA overexpression is an early event in carcinogenesis. These data, taken together, suggest that the expression of TCSF mRNA can be correlated with tumor progression. However, despite its implication in cancer progression, TCSF might play a role in some other pathological processes, such as inflammation and emphysema, because it has also been detected in alveolar macrophages in our study and it has been proposed as a factor in arthritis (Karinrerk et al. 1992).

Even though the precise function of TCSF is not known, some recent in vitro studies have shown that TCSF is able to stimulate the production of several MMPs by fibroblasts. Recent experimental data have demonstrated that TCSF stimulates the production of interstitial collagenase, stromelysin 1 and gelatinase A but not stromelysin 3 in fibroblasts (Kataoka et al. 1993). However, stromelysin 3 is known to have a poor proteolytic activity against collagen-like substrates (Murphy et al. 1994). Moreover, these authors have also demonstrated that TCSF increases activation of gelatinase A. These results are of particular interest regarding the implication of TCSF in cancer progression, because in many carcinomas in vivo the stromal cells have been demonstrated to be the principal source of several MMPs. A variety of studies have indicated that fibroblasts adjacent to the malignant tumors clusters produce interstitial collagenase (Urbanski et al. 1992; Hewitt et al. 1991; Polette et al. 1991), stromelysins 1, 2, 3 (Polette et al. 1991; Basset et al. 1990), and MT-MMP1 (Polette et al. 1996; Okada et al. 1995). More precisely, in both lung and breast carcinomas, mRNAs encoding gelatinase A, which degrades basement membrane collagens (Tryggvason et al. 1993), have also been localized by ISH in the stromal cells surrounding invasive carcinomas (Polette et al. 1993,1994; Poulsom et al. 1992,1993; Soini et al. 1993). In addition to their specific localization at the tumor–stromal interface, MMPs were not or were only weakly found in normal tissues and benign lesions. These studies have therefore demonstrated an association between MMP expression and the invasive process in cancers. Using serial sections in breast carcinomas, we showed that there is an obvious expression of TCSF mRNAs by tumor cells and gelatinase A mRNAs by fibroblasts in the same areas.

It therefore appears that some MMPs, as well as TCSF, are expressed selectively in both pre-invasive and invasive carcinoma but by different cell types, peritumoral fibroblasts and tumor cells, respectively. Relating our in vivo data to the observation that TCSF

enhances the production of some particular MMPs in fibroblasts in vitro, it can be postulated that the TCSF produced by tumor cells in vivo stimulates the expression of some MMPs by peritumoral fibroblasts. However, in vivo, interstitial collagenase and stromelysin 1, which are induced by TCSF in fibroblasts in vitro, are infrequently observed in stromal cells in breast carcinomas (Polette et al. 1991), whereas they are present at high levels in lung cancers (Muller et al. 1991), despite the presence of TCSF in both types of carcinoma. Moreover, in four of 22 of our lung carcinomas, we failed to detect any TCSF transcripts. These findings are consistent with the involvement of other factors in the regulation of those MMPs, such as the origin of the tissue, the extracellular matrix environment, and genetic rearrangements.

In conclusion, our observations on lung and breast cancers strongly support the hypothesis that TCSF is an important factor in tumor progression. More precisely, TCSF produced by tumor cells could play a role in the degradation of extracellular matrix associated with tumor invasion by stimulating the synthesis of some MMPs by peritumoral fibroblasts.

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Stimulation of Matrix Metalloproteinase Production by Recombinant Extracellular Matrix Metalloproteinase Inducer from Transfected Chinese Hamster Ovary Cells*

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Many of the tumor-associated matrix metalloproteinases that are implicated in metastasis are produced by stromal fibroblasts within or surrounding the tumor in response to stimulation by factors produced by tumor cells. In this study we transfected Chinese hamster ovary cells with putative cDNA for human extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein that is attached to the surface of many types of malignant human tumor cells and that has previously been implicated in stimulation of matrix metalloproteinase production in fibroblasts. We show that these transfected cells synthesize EMMPRIN that is extensively post-translationally processed; this recombinant EMMPRIN stimulates human fibroblast production of interstitial collagenase, stromelysin-1, and gelatinase A (72-kDa type IV collagenase). We propose that EMMPRIN regulates matrix metalloproteinase production during tumor invasion and other processes involving tissue remodeling.

Successful tumor metastasis requires many steps, one of which is local proteolytic destruction of extracellular matrix at sites of tumor invasion. A major class of proteinases associated with tumor invasion is the matrix metalloproteinases (MMPs)[1] (1, 2). Although it was initially thought that these enzymes were mainly produced by malignant tumor cells themselves, it is now clear that interstitial collagenase (MMP-1), gelatinase A (MMP-2, a 72-kDa type IV collagenase), and stromelysin-1 (MMP-3) are produced *in vivo* by stromal fibroblasts associated with several types of tumors (2–7). MMP-2 synthesized and secreted by these fibroblasts has been shown to adhere to the surface of tumor cells, facilitating tissue invasion (8–10). Because quiescent fibroblasts generally produce relatively low amounts of MMPs (11, 12), tumor-associated fibroblasts must be influenced in some way to give rise to the elevated levels of MMPs usually present in malignant tumors. One possibility that we have investigated is that tumor cells interact with fibroblasts via soluble or cell-bound factors, stimulating fibroblast MMP production (11–15). Our studies have led to characterization of a tumor cell surface protein, extracellular matrix metalloproteinase inducer (EMMPRIN; previously termed tumor cell-derived collagenase stimulatory factor or TCSF), that stimulates fibroblast production of MMP-1, MMP-2, and MMP-3 (12–14). We recently obtained cDNAs for human EMMPRIN and verified their identity by recognition of recombinant EMMPRIN by activity-blocking monoclonal antibody and by sequence identity with amino acid sequences of peptides isolated from EMMPRIN (15). However, recombinant EMMPRIN produced by bacteria is much smaller than native EMMPRIN isolated from tumor cells because it is not post-translationally processed. This form of recombinant EMMPRIN is inactive, thus leaving some doubt regarding the identity of the cDNAs. In this study, we use the cDNAs to transfect CHO cells and show that EMMPRIN produced by these transfected cells is post-translationally processed and stimulates fibroblasts to produce elevated levels of MMP-1, MMP-2, and MMP-3.

EXPERIMENTAL PROCEDURES

Stable Transfection of CHO Cells with EMMPRIN cDNA—EMMPRIN cDNA (15) was subcloned into an expression vector, pcDNA/Neo (Invitrogen, San Diego, CA), and purified by CsCl gradient centrifugation and phenol/chloroform extraction. CHO cells (American Type Culture Collection, Bethesda, MD) were seeded at 10^6 cells/100-mm tissue culture dish and incubated overnight, at which stage they were 50–70% confluent. The cells then were transfected in 5 ml of serum-free Ham's F-12 medium containing lipofectamine-DNA complex (10 μl of lipofectamine (Life Technologies, Inc.) mixed with 10 μg of DNA with or without the EMMPRIN insert). After 6 h of incubation at 37 °C, 5 ml of medium containing 20% fetal bovine serum was added to the transfection mixture, which was then cultured at 37 °C for a further 72 h. The cells then were treated with trypsin-EDTA (Life Technologies, Inc.) and subcultured in medium containing 400 mg/liter of Geneticin (Life Technologies, Inc.) for 2–3 weeks. Successful transfection was assessed by immunocytochemistry using monoclonal antibody E11F4 raised against EMMPRIN, as described previously (13).

Purification of EMMPRIN—EMMPRIN was purified from detergent extracts of cell membranes from LX-1 cells or stably transfected CHO cells by immunoaffinity chromatography using monoclonal antibody E11F4 against EMMPRIN as described previously (13). Briefly, the cell membranes were extracted with 10 mM Tris-HCl buffer (pH 8.2), containing 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The supernatant of the extract was then applied to a 5-ml anti-EMMPRIN affinity column and recirculated through the column for 12 h at 4 °C. The column was washed with buffer several times, and EMMPRIN was then eluted from the column with 50 mM diethylamine, 30 mM octylglucoside (pH 11.5). The eluted protein was neutralized with 0.5 M NaH_2PO_4, dialyzed against 0.1 M acetic acid, concentrated, and dissolved in 0.1 M acetic acid.

Assays for EMMPRIN Activity—Human fibroblasts (isolated from

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† This article is dedicated to the memory of our friend and colleague, Chitra, in whose laboratory much of this work was done but who died in August, 1993.

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[1] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate ester; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

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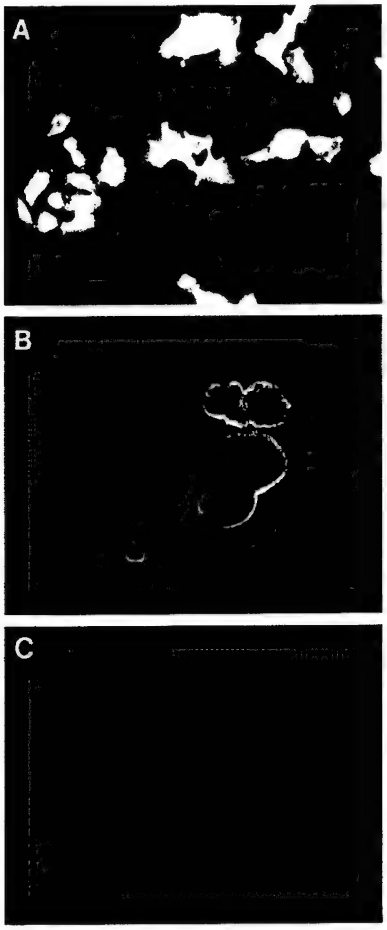

FIG. 1. **Immunofluorescent staining of CHO cells transfected with EMMPRIN cDNA.** Staining was carried out using monoclonal antibody, E11F4, against EMMPRIN as described previously (13). *A*, CHO cells transfected with EMMPRIN cDNA, fixed under normal culture conditions. *B*, similar cells to those in *A*, but fixed 4 h after plating. *C*, CHO cells mock-transfected with vector. Untransfected CHO cells also show no reactivity with E11F4 (not shown).

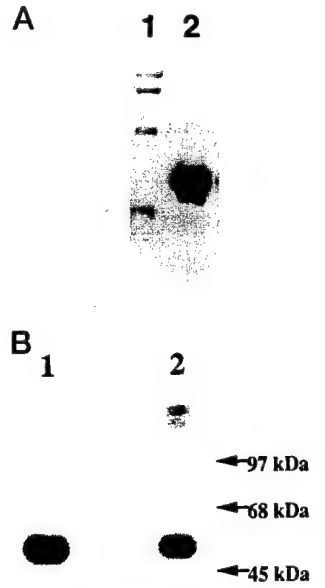

FIG. 2. **SDS-PAGE and Western blotting of purified recombinant EMMPRIN.** *A*, silver-stained SDS-PAGE gel of EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA. EMMPRIN was purified from cell membranes as described under "Experimental Procedures," dissolved in SDS sample buffer containing 0.1 M dithiothreitol, heated at 95 °C for 10 min, and subjected to 10% SDS-PAGE; the gel was deliberately overloaded to reveal potential contaminants. *Lane 1*, molecular mass standards (45, 66, 97, and 116 kDa); *lane 2*, purified recombinant EMMPRIN. *B*, Western blot of recombinant EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA (*lane 1*) and of native EMMPRIN purified from LX-1 cells (*lane 2*). A 10% SDS-PAGE gel was electroblotted to a nitrocellulose membrane followed by blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20. The blot was incubated with E11F4 hybridoma supernatant (13) for 1 h at room temperature and then with horseradish peroxidase-conjugated anti-mouse IgG. The EMMPRIN protein bands were detected with ECL Western blotting detection reagents (Amersham Corp.). In both cases the anti-EMMPRIN antibody recognized a protein with a molecular mass of ~58 kDa. Some immunoreactive, aggregated protein was also present in LX-1 cells, as previously noted (14).

human skin in our laboratory) were cultured for 24 h in 24-well plates in 1 ml of DMEM medium supplemented with 10% fetal bovine serum, after which the medium was replaced with 0.5 ml of DMEM containing 2% fetal bovine serum in the presence or the absence of EMMPRIN or TPA, and the cultures were further incubated at 37 °C for 3 days. Media from these cultures were used for zymographic assay of MMP-3 (16) and ELISA of MMP-1, MMP-2, and MMP-3 (12, 17).

RESULTS

In initial attempts to demonstrate recombinant EMMPRIN activity we tested purified, pGEX bacterial expression protein. However, EMMPRIN produced in the pGEX system had a molecular mass of only ~29 kDa (equivalent to that expected from the cDNA open reading frame (15)), compared with native EMMPRIN from tumor cells, which is ~58 kDa (12–14). This bacterially produced recombinant EMMPRIN protein was inactive in stimulating MMP production by human fibroblasts. Next, COS and CHO cells were transfected with EMMPRIN cDNA under a variety of conditions, but in most cases the EMMPRIN produced was either of similar molecular mass to bacterial recombinant protein, *i.e.* ~29 kDa, or was partially post-translationally processed with molecular masses ranging from 30–45 kDa. EMMPRIN isolated from these cells was also inactive.

However we found that after stable transfection (see "Experimental Procedures"), CHO cells could be selected that synthesize high levels of EMMPRIN of similar molecular mass to that of native EMMPRIN isolated directly from LX-1 human carcinoma cells. Fig. 1*A* shows detection of this recombinant EMMPRIN in the transfected CHO cells by immunocytochemistry using monoclonal antibody raised against native EMMPRIN from LX-1 cells (13). Because the transfected cells are very flat, it is difficult to discern the precise cellular distribution of EMMPRIN. However, if the cells are fixed shortly after plating (~4 h), *i.e.* before they have flattened, it is clear that EMMPRIN is located at the surface of the transfected cells (Fig. 1*B*). Untransfected cells or cells that are mock-transfected with vector only show no reactivity with the antibody (Fig. 1*C*).

Fig. 2*A* shows a silver-stained SDS-PAGE gel of recombinant EMMPRIN purified by immunoaffinity chromatography from membrane extracts of the stably transfected CHO cells; this gel was deliberately overloaded to reveal potential contaminants in the preparation. A single broad band at ~58 kDa was detected, as previously obtained for tumor cell-derived EMMPRIN (13, 14). Direct comparison of purified recombinant and LX-1 carcinoma cell-derived EMMPRIN by Western blotting showed that they were identical in size (Fig. 2*B*), indicating that the recombinant EMMPRIN was fully or almost fully post-translationally processed. Untransfected CHO cells or cells transfected with vector only did not produce any EMMPRIN detectable by immunoaffinity chromatography and Western blotting (not shown).

We then tested purified recombinant EMMPRIN from transfected CHO cells for its ability to stimulate MMP production by human fibroblasts in culture. We first measured the effect of recombinant EMMPRIN on MMP-3 production by zymography,

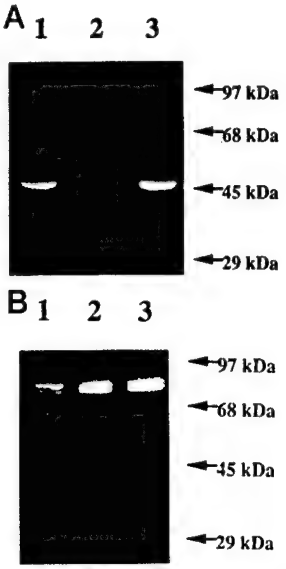

FIG. 3. **Stimulation of stromelysin-1 (MMP-3) production in human fibroblasts incubated with recombinant EMMPRIN isolated from transfected CHO cells.** Media (15 μl each) from fibroblasts cultured in the presence or the absence of EMMPRIN or TPA were subjected to electrophoresis without reduction in 10% SDS-polyacrylamide impregnated with 0.5 mg/ml β-casein (*A*) or 0.5 mg/ml carboxymethylated transferrin (*B*). After electrophoresis, the SDS was eluted from the gels in 2.5% Triton X-100 for 30 min at 25 °C, and the gels were then incubated 20 h in substrate buffer containing 50 mM Tris-HCl and 5 mM $CaCl_2$. The gels were stained with Coomassie Blue, and the presence of proteolytic enzymes was identified by the appearance of clear zones where the casein or transferrin substrate had been digested. *Lane 1*, fibroblasts incubated with recombinant EMMPRIN (100 μg/ml); *lane 2*, fibroblasts incubated without added protein; *lane 3*, fibroblasts incubated with TPA (0.1 μg/ml). The clear band at ~45 kDa present in *lanes 1* and *3*, but in much lower amount in *lane 2*, represents MMP-3. The other clear zones are due to other proteolytic enzymes constitutive to the fibroblasts.

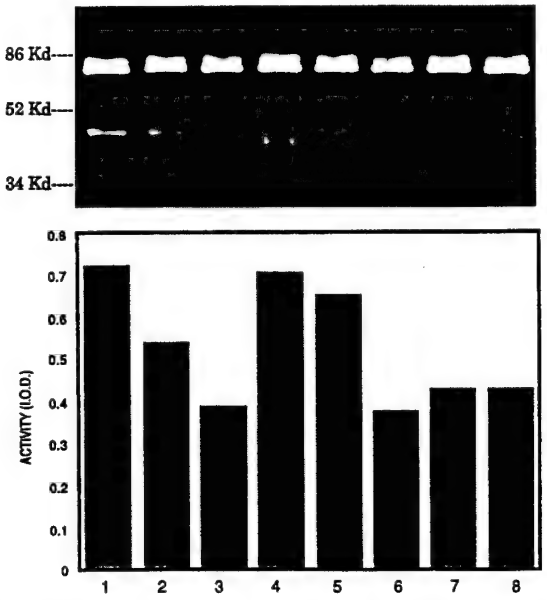

FIG. 4. **Effect of antibody against native EMMPRIN on stimulation of MMP-3 production by recombinant EMMPRIN.** MMP-3 was detected in the same fashion as described in the legend to Fig. 3*B* using 0.5 mg/ml of carboxymethylated transferrin as substrate. The *top panel* shows the zymogram; the *bottom panel* shows quantitation of the ~45-kDa band (or doublet) in each lane by densitometry using a Bio Image Whole Band Analysis package. *Lane 1*, plus 100 ng/ml TPA; *lane 2*, plus 50 ng/ml TPA; *lane 3*, no addition; *lanes 4* and *5*, plus 100 μg/ml recombinant EMMPRIN; *lane 6*, plus supernatant derived after protein A treatment of medium containing 100 μg/ml recombinant EMMPRIN and EIIF4 monoclonal antibody against EMMPRIN, as described previously (13); *lane 7*, plus 100 μg/ml recombinant EMMPRIN and antibody EIIF4; *lane 8*, no addition.

using two separate substrates, casein (Fig. 3*A*) and carboxymethylated transferrin (Fig. 3*B*). A clear-cut increase in active MMP-3 was observed in fibroblasts treated with the recombinant EMMPRIN (Fig. 3, *A* and *B*, *lane 1 versus 2*). The amount of MMP-3 was similar to that induced by TPA treatment (Fig. 3, *A* and *B*, *lane 3*).

To ensure that stimulation of MMP-3 production was not due to minor contaminants in the recombinant EMMPRIN preparation, we tested the effect of blocking antibody raised against native EMMPRIN (13) on the stimulation by recombinant EMMPRIN, using two different approaches. In the first approach, antibody was included in the culture medium together with EMMPRIN throughout the 3-day incubation period (Fig. 4, *lane 7*). In the second approach, the antibody was mixed with EMMPRIN and then removed by binding to protein A; the supernatant from this reaction, depleted of antigen, was then added to the culture for the 3-day incubation (Fig. 4, *lane 6*). As shown in Fig. 4, the stimulation of stromelysin production in cells treated with EMMPRIN (*lanes 4* and *5 versus lanes 3* and *8*) was completely reversed by either of the two different treatments with antibody to EMMPRIN (*lanes 6* and *7*).

Finally, we measured the effect of recombinant EMMPRIN on MMP-1, MMP-2, and MMP-3 production by ELISA. In two separate experiments, treatment of fibroblasts with the EMMPRIN gave rise to significant increments in production of MMP-1 (~6- and ~11-fold), MMP-2 (~1.5- and ~16-fold), and MMP-3 (~2- and ~4-fold) (Table I). In most cases the degree of stimulation of MMP by recombinant EMMPRIN was similar to that caused by TPA (Table I).

DISCUSSION

The results presented here constitute final proof that the cDNAs that we have obtained genuinely encode EMMPRIN, as defined by its MMP stimulatory activity. The degree of stimulation of MMP-1, MMP-2, and MMP-3 by recombinant EMMPRIN obtained herein is similar to that obtained previously with native EMMPRIN purified from LX-1 carcinoma cells (12). It should be pointed out, however, that the degree of stimulation by either native or recombinant EMMPRIN varies in different fibroblast preparations. First, some fibroblast preparations are not significantly responsive to EMMPRIN stimulation, whereas others are very responsive (*e.g.* see Ref. 12). Second, among populations of fibroblasts that are responsive to EMMPRIN, an important variable is the extent to which a batch of fibroblasts already produces a given MMP without addition of stimulatory agents. This can be readily appreciated for MMP-3 production in Fig. 3*B*, *lane 2*, versus Fig. 4, *lanes 3* and *8*, and for MMP-2 production in Table I, experiment 1 *versus* experiment 2. Our overall experience has been that EMMPRIN stimulation of MMP-1 and MMP-3 production is usually in the range of 3–10-fold. Stimulation of MMP-2, however, is even more variable (*e.g.* see Table I, experiment 1 *versus* 2) but is usually rather modest, *e.g.* 1.5–2-fold. One reason for this variability is clearly the significant amounts of MMP-2 that many fibroblast preparations produce without treatment with exogenous agents, *e.g.* experiment 1 in Table I. Also, however, the mechanism of stimulation of MMP-2 may be more complex than for MMP-1 and MMP-3. As well as stimulating overall MMP-2 production, EMMPRIN apparently enhances activation of MMP-2, and this sometimes leads to underestimation of MMP-2 stimulation in ELISA assays (12).

The experiments described here indicate that EMMPRIN activity is dependent on post-translational processing. How-

TABLE I

Stimulation of MMP production by recombinant EMMPRIN

Recombinant EMMPRIN was purified from membranes of transfected CHO cells and, in two separate experiments, added at 100 μg/ml to cultures of human fibroblasts. Cultures were also incubated with TPA (0.1 μg/ml) or with no added reagent. After incubation, aliquots of culture medium were used for ELISA of MMP-1, MMP-2, and MMP-3. Amounts of MMP are expressed as μg/ml ± S.E.

Agent added	MMP-1	MMP-2	MMP-3
Experiment 1			
None	0.03 ± 0.00	1.40 ± 0.01	0.21 ± 0.01
rEMMPRIN	0.33 ± 0.02[a]	2.12 ± 0.13[a]	0.93 ± 0.13[a]
TPA	0.32 ± 0.02[a]	2.33 ± 0.29[a]	0.42 ± 0.02[a]
Experiment 2			
None	0.03 ± 0.00	0.13 ± 0.01	0.35 ± 0.04
rEMMPRIN	0.17 ± 0.02[a]	2.10 ± 0.37[a]	0.63 ± 0.03[a]
TPA	0.25 ± 0.02[a]	0.46 ± 0.06[a]	0.56 ± 0.07[a]

[a] Significantly greater than control (none added), $p < 0.05$.

ever, it is not yet clear whether processing is required to attain the appropriate conformation for activity or whether specific side groups, *e.g.* carbohydrate or phosphate, are involved in EMMPRIN receptor recognition.

We have shown by immunocytochemistry that EMMPRIN is present on the surface of tumor cells but not fibroblasts and several other normal adult cell types (13, 18, 19). Prior studies have also shown that tumor cells shed EMMPRIN (13) and that EMMPRIN appears in the urine of bladder carcinoma patients (18, 20). Thus tumor cell EMMPRIN, in soluble or membrane-bound form, is likely to be responsible for at least part of the stimulation of fibroblast MMP production observed *in vivo* in association with a variety of malignant tumors (3–7). In preliminary experiments, we have found that production of the tissue inhibitor of matrix metalloproteinases, TIMP-1, is not stimulated by EMMPRIN; if this proves to be true *in vivo*, an imbalance of active *versus* inactive MMP production may result from EMMPRIN action on stromal fibroblasts.

The amino acid sequence of EMMPRIN is identical to that of human M6 antigen (21) and basigin (22), for which no function had previously been ascribed until EMMPRIN was cloned (15). Several proteins with high levels of homology to EMMPRIN, *i.e.* neurothelin, HT7, OX47, and gp42, have also been characterized in other species (21–23), but again their function has not been determined. These proteins may be species homologues of one another or closely related members of a protein family; it remains to be seen whether all of these proteins have EMMPRIN activity.

The ability of EMMPRIN to stimulate MMP production has led us to propose that it may be involved in a wide range of physiological and pathological processes where tissue remodeling takes place (24). For example, its presence in the epidermis (25) and in several embryonic epithelia[2] suggests that EMMPRIN may participate in epithelial-mesenchymal interactions leading to changes in tissue architecture during development and wound healing. Also, EMMPRIN on the surface of activated lymphocytes and monocytes (21) may contribute to elevated MMP levels found in arthritis. However, association of EMMPRIN-like material with endothelium during formation of the blood-brain barrier and with highly organized epithelia such as retina and kidney tubules (23) suggests that it may have additional functions involving cell-cell interactions.

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Characterization of the gene for human EMMPRIN, a tumor cell surface inducer of matrix metalloproteinases

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Abstract

EMMPRIN (extracellular matrix metalloproteinase inducer) also known as CD147 and basigin, is a member of the immunoglobulin family that is present on the surface of tumor cells and stimulates nearby fibroblasts to synthesize matrix metalloproteinases. Using our EMMPRIN cDNA, we have isolated a cosmid clone that contains the human EMMPRIN gene. S1 analysis with a fragment of the gene clone and primer extension of the mRNA was performed to determine the transcription start site. PCR and sequence analysis have defined the exon/intron organization of the gene and show that it is highly conserved with the mouse EMMPRIN/basigin gene. About 950 bases of the 5'-flanking region were examined for transcription factor consensus binding sites, locating three SP1 sites and two AP2 sites. The transcription start site was found to be located in a CpG island. Elements in the proximal promoter region were conserved in the human and mouse genes. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Tumor cell-derived collagenase stimulatory factor; basigin; M6; CD147

1. Introduction

EMMPRIN (also called CD147, basigin or M6 in the human) is a member of the immunoglobulin superfamily, which includes T cell receptors, neural cell adhesion molecules and major histocompatibility complex antigens. Several groups (Altruda et al., 1989; Ellis et al., 1989; Fadool and Linser, 1993; Kasinrerk et al., 1992; Miyauchi et al., 1990; Nehme et al., 1993; Seulberger et al., 1992) independently discovered this glycoprotein in a variety of species, following different experimental pathways. In the first functional approach, the Biswas laboratory discovered EMMPRIN, then called tumor cell-derived collagenase stimulatory factor (TCSF), as a surface molecule on tumor cells that stimulated nearby fibroblasts to produce matrix metalloproteinases (Biswas et al., 1995; Ellis et al., 1989; Guo et al., 1997; Kataoka et al., 1993). This intercellular recognition function is consistent with the known functions of the immunoglobulin superfamily.

cDNA hybridizations have been used to determine the chromosomal location of both the mouse and human EMMPRIN/basigin genes, mapping the mouse to chromosome 10 (Simon-Chazottes et al., 1992) and the human to 19p13.3 (Kaname et al., 1993). The gene has been disrupted in a transgenic mouse line. Most transgenic embryos lacking the EMMPRIN/basigin gene die around the time of implantation (Igakura et al., 1996; Igakura et al., 1998), a time when intercellular recognition events and matrix metalloproteinase involve-

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[1] Chitra Biswas, our Tufts colleague who started this work, died 26 August 1993.

Abbreviations: AMV, avian myeloblastosis virus; bp, base pair(s); cDNA, DNA complementary to RNA; CpG, deoxycytidyl-deoxyguanosyl dinucleotide; dUTP, deoxyuridyl triphosphate; EDTA, [*N*, *N*, *N'*, *N'*]-ethylenediaminetetraacetic acid; EMMPRIN, extracellular matrix metalloproteinase inducer; FISH, fluorescence in situ hybridization; fmol, femtomol or 10^{-15} mole; Ig, immunoglobulin; kb, kilobase; nt, nucleotide(s); PCR, polymerase chain reaction; rc, reverse complement; SDS, sodium dodecyl sulfate; SSPE, standard saline phosphate-EDTA; TCSF, Tumor cell-derived collagenase stimulatory factor; UTR, untranslated region.

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ment are critical (Alexander et al., 1996). In addition, mouse genomic clones have been characterized (Cheng et al., 1994; Miyauchi et al., 1995). In this report we present the first isolation and characterization of the human gene. We have determined the transcription start site and elucidated the exon/intron structure. While a high degree of conservation is seen between the human and mouse gene structures, a notable difference is that the human protein is encoded by eight exons, while that of the mouse is encoded by seven. We have also sequenced approx. 950 bp of the 5′-flanking region of the EMMPRIN gene and have searched it for transcription factor binding site consensus sequences.

2. Materials and methods

2.1. Isolation of the EMMPRIN genomic clone

Filter lifts of a human placental genomic library in cosmid vector pWE15 (Stratagene, La Jolla, CA, USA) were screened with the full-length EMMPRIN cDNA (Biswas et al., 1995), radiolabeled by nick translation. A positively hybridizing candidate cosmid was isolated and expanded. Two criteria were used to verify the cosmid's identity. The first was partial sequence analysis, which indicated that some EMMPRIN sequences were contained within the cosmid clone. The second verification method was fluorescent in situ hybridization (FISH), with biotinylated cosmid clone as probe. Biotin-11-dUTP was incorporated into the probe using the Bio-Nick nick translation kit (Life Technologies, Grand Island, NY, USA), following the manufacturer's instructions. The probe was hybridized in situ to BrdU-synchronized normal female lymphocytes (Lemieux et al., 1992; Lichter et al., 1990), followed by reaction with fluorescein goat anti-biotin and fluorescein-labeled anti-goat IgG (Vector Laboratories, Inc. Burlingame, CA.). Visualization of hybridization to metaphase chromosomes was accomplished by epifluorescence microscopy. The selected cosmid localized only to chromosome 19p13.3 and was used for all subsequent analyses.

To isolate a manageable piece of DNA from the cosmid clone for further analysis, the cosmid DNA containing the EMMPRIN gene was digested with several restriction enzymes. Fragments were separated by electrophoresis on a 1% agarose gel, followed by transfer to nylon membrane. This was hybridized with a ^{32}P-end-labeled 25-mer oligonucleotide probe derived from the EMMPRIN cDNA (CAGCGCGA-ATCCCAGCAGCACGAAC, positions 52–76 in Fig. 1). After hybridization, the blot was washed twice at 65°C with 1×SSPE, 0.1% SDS, dried and exposed to film. A 3 kb *Bam*HI/*Eco*RI double digestion fragment hybridized to the probe. Another aliquot of cosmid DNA was double digested and the 3 kb band was purified from an agarose gel, then subcloned in pBluescript vector (Stratagene). The sequence of this genomic fragment was generated using a Bst DNA sequencing Kit (Bio-Rad, Hercules, CA, USA).

2.2. mRNA isolation, primer extension and S1 nuclease analyses

Total RNA was prepared from LX-1 cells as described previously (Biswas et al., 1995). To determine the transcription start site (the beginning of exon 1), two genomic fragments were prepared that contained the 5′-end of the cDNA (estimated to be approx. 100 nucleotides short of a full-length copy of the mRNA) plus several hundred bases upstream, for hybridizing to the RNA prior to S1 nuclease digestion. The two genomic fragments, 331 nt (−263 to +68 in Fig. 1) and 492 nt (−424 to +68), were amplified from the cosmid template by PCR using the following primers: as 3′-primer for generating both fragments, GATGATGATATC-CCAGCAGCACGAACAG, (an *Eco*RV recognition sequence [in bold], preceded by 6 nt to ensure that the enzymatic site would not be at an end where local strand separation occurs, preventing cleavage, and followed by the reverse complement of nucleotides 49–68 (Fig. 1): bases 66–68 (Fig. 1) overlap the restriction enzyme recognition site); for the smaller product the 5′-primer was nucleotides −263 to −244 (Fig. 1 and Fig. 2A); for the larger product the 5′-primer was −424 to −403 (Fig. 1 and Fig. 2A). These 5′-primers are double underlined in Fig. 1. The PCR products were subcloned into pCRII (Invitrogen, San Diego, CA, USA), and used to transform bacteria. Colonies were grown and selected, and isolated plasmid DNAs were digested with *Eco*RV, and applied to a 1% agarose gel. The linearized products that were extracted from the gel were end-labeled with ^{32}P by T4 polynucleotide kinase. Since the subcloned genomic sequences contained no *Bam*HI sites, but such a site was present in the vector at a site distal from the *Eco*RV site, a subsequent cleavage with *Bam*HI released the entire genomic insert attached to a piece of the vector. In addition, the double digestion ensured that only the strand of genomic DNA complementary to the mRNA contained the label. The large and small double digestion products were gel purified and used for S1 nuclease protection using an S1 Assay kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. Briefly, 50 μg of total RNA was mixed with 50 000 cpm of each probe in hybridization buffer (80% formamide, 100 mM sodium citrate, 300 mM sodium acetate, 1 mM EDTA, pH 6.4), heated at 90°C for 5 min and hybridized at 42°C overnight. The hybridization mixtures were then digested with 50 unit of S1 nuclease for 30 min at 37°C. Following ethanol precipitation, the digested products were resus-

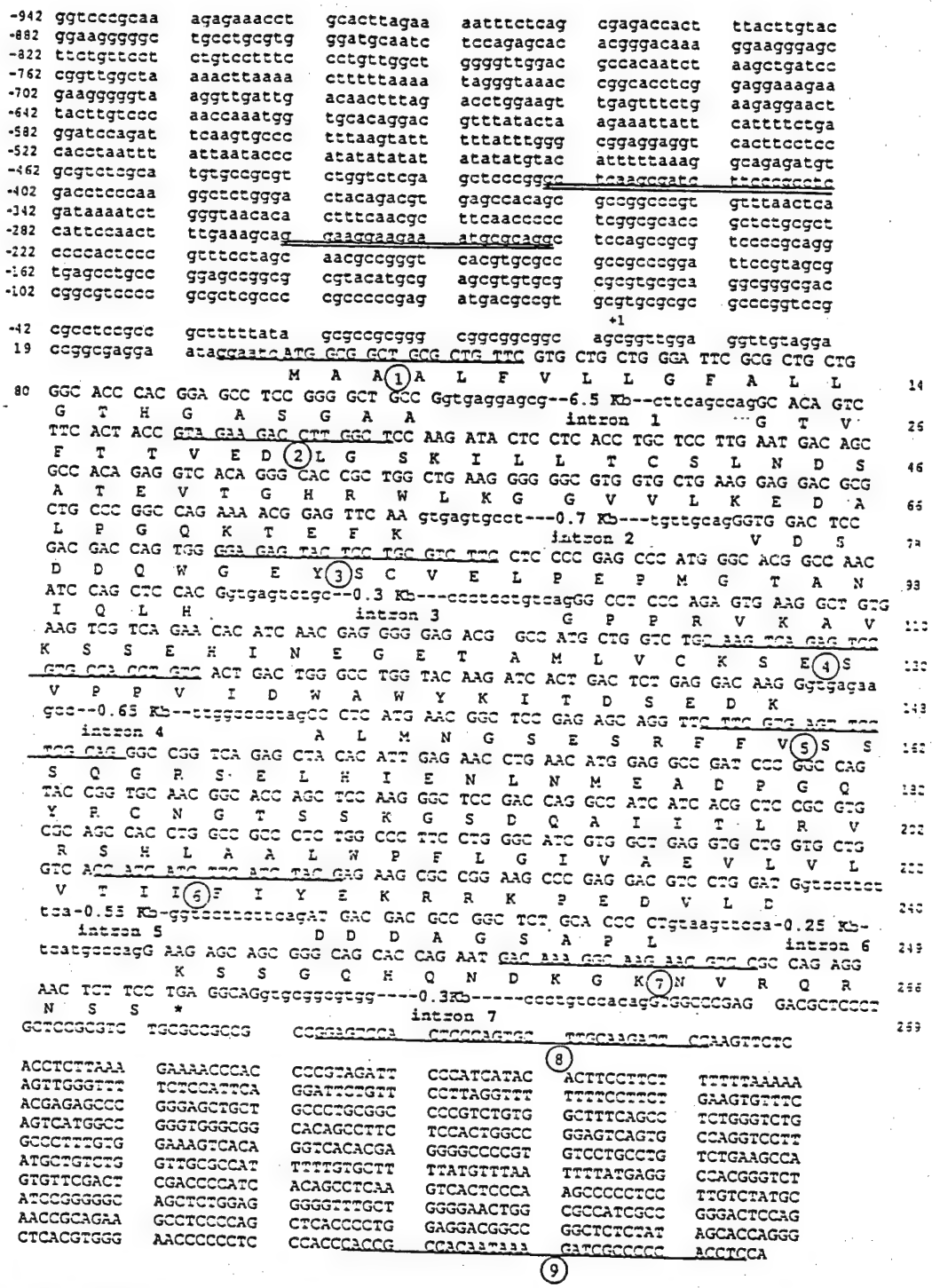

Fig. 1. Sequence of the EMMPRIN gene and 5′-flanking region. A +1 is shown above the transcription start site. The sequence includes 942 bases 5′ of the transcription start site, all the exonic sequences, and the borders of the introns. The 5′-primers used to generate S1 nuclease probes are indicated by double underlines. Primers used to determine the exon/intron organization are single underlined with circled numbers below them. Exonic sequences are in upper-case letters: intronic and 5′-flanking sequences are in lower-case letters. The stop codon is indicated with an asterisk.

pended in loading buffer (95% formamide, 0.5 mM EDTA, 0.025% SDS, 0.025% xylene cyanol, 0.025% bromophenol blue) and resolved on a 5% polyacrylamide–8 M urea denaturing gel. The gel was dried and exposed to X-ray film.

A negative and positive control probe were also used in the S1 nuclease analysis. Since the Northern blot estimated the mRNA size as approx. 1.6 kb, about 100 bases longer than the cDNA, we reasoned that regions in the gene more than 100 bases upstream of the coding

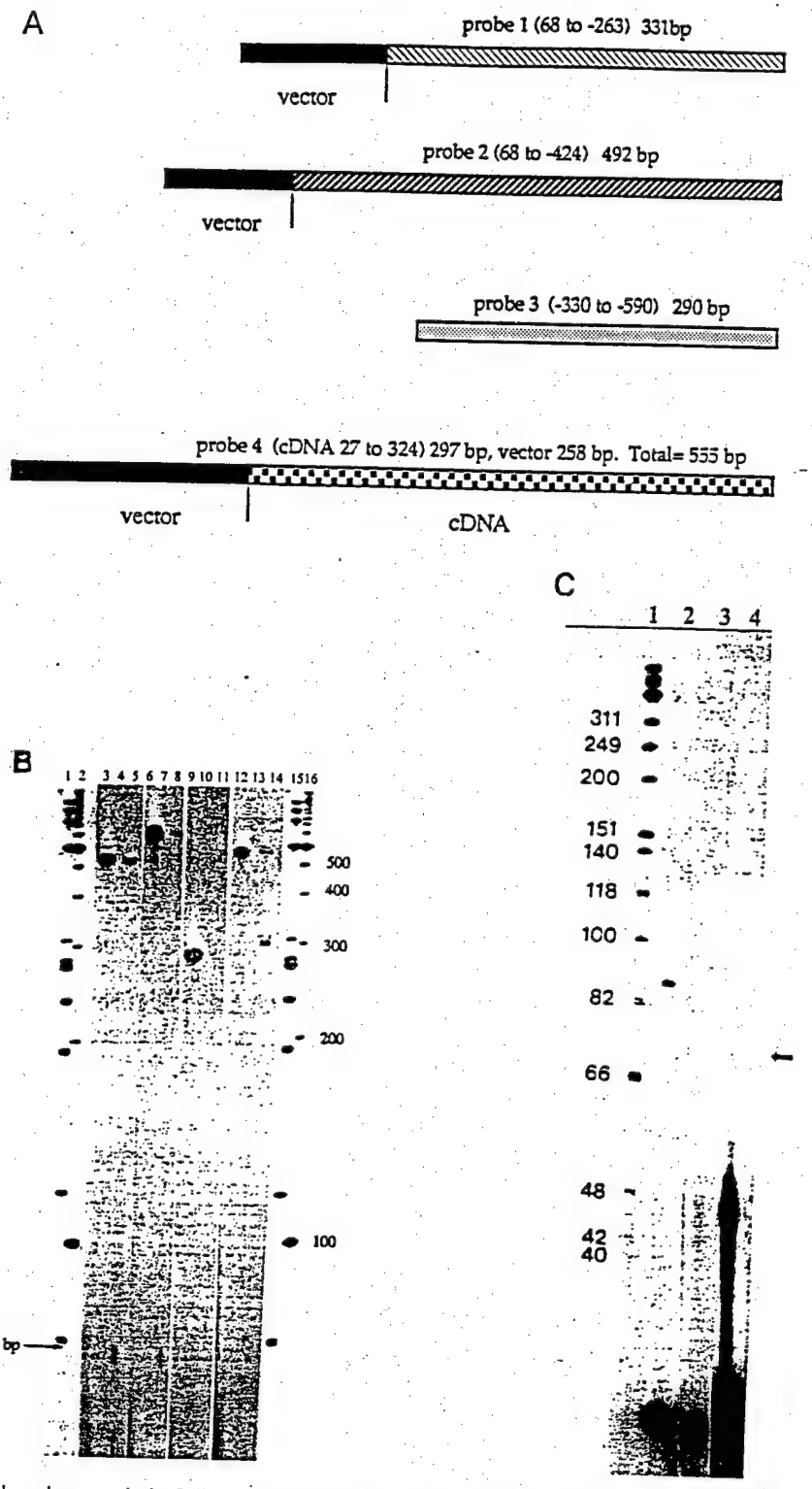

Fig. 2. (A) Probes used for S1 nuclease analysis. Solid black lines indicate vector portions of the probes. Numbering refers to Fig. 1. Probe 3 is a negative control; probe 4 is a positive control. (B) S1 nuclease analysis to determine the transcription start site. Lanes 1 and 15, radiolabeled size marker ϕX174 digested with *Hae*III; lanes 2 and 16, radiolabeled 100 bp ladder size marker; lane 3, the 331 bp EMMPRIN probe with approx. 200 bp vector attached; lane 4, the vector/331 bp probe digested with S1 nuclease; lane 5, the vector/331 bp probe hybridized to mRNA, then digested with S1 nuclease; lane 6, the 492 bp probe with approx. 200 bp vector attached; lane 7, the vector 492 bp probe digested with S1 nuclease; lane 8, the vector/492 bp probe hybridized to mRNA, then digested with S1 nuclease; lane 9, the negative control probe; lane 10, the negative control probe digested with S1 nuclease; lane 11, the negative control probe hybridized to mRNA, then digested with S1 nuclease; lane 12, the positive control probe derived from an internal region of the EMMPRIN cDNA; lane 13, the positive control probe digested with S1 nuclease; lane 14, the positive control probe hybridized to mRNA, then digested with S1 nuclease. Lanes 5 and 8 show that a 68 bp fragment (arrow) of

region would not be in the mRNA and could serve as a negative control. Therefore, a labeled PCR product corresponding to gene nucleotides −330 to −590 (Fig. 1) was labeled as a negative control probe. A positive control, a restriction fragment of a partial cDNA clone, was employed to assure us that the S1 nuclease protection analysis was working perfectly, i.e. that only protected products were observed, one being the 555 bp reannealed probe (since the probes are double-stranded DNA, a portion of the probe is expected to reanneal to itself, thereby preventing digestion), and the other being the probe fragment protected by mRNA. This positive control probe contained the 5′-most 297 bases of our partial copy cDNA (the nucleotide numbering refers to Fig. 1—plus numbers are exonic sequences only), attached to 258 bp of vector (totaling 555 bp, see Fig. 2A).

For primer extension analysis, a Promega Primer Extension System kit was used. 10 pmol of control primer or EMMPRIN antisense primer (GAATCCCAGCAGCACGAACAGCGCA: reverse complement of nucleotides 46–70) were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase for 10 min at 37°C. The reactions were inactivated by heating to 90°C for 2 min, and the primer concentration was adjusted to 100 fmol/μl with water. 100 fmol of each labeled primer was aliquotted for the following ethanol precipitations: with control primer alone (no RNA), with control primer plus 1 μg kit control RNA, with EMMPRIN antisense primer alone (no RNA), and with EMMPRIN antisense primer plus 1 μg LX-1 mRNA. The pellets were resuspended in 10 μl hybridization buffer (80% formamide, 1 mM EDTA, 100 mM sodium citrate, 300 mM sodium acetate, pH 6.4), heated to 95°C for 5 min, then allowed to hybridize overnight at 42°C. These mixtures were again ethanol precipitated then resuspended with 10 μl AMV primer extension buffer (Promega, Madison, WI, USA) containing 1 unit of AMV reverse transcriptase. After incubation for 90 min at 42°C, 10 μl loading buffer (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) were added and the mixture was heated to 90°C for 10 min. 10 μl of each sample was electrophoretically separated on a 10% denaturing polyacrylamide gel. The gel was dried and exposed to film.

2.3. Determination of exon/intron junctions

Several steps were employed to elucidate the EMMPRIN gene structure. First, to determine the overall size of the gene, a primer derived from the 5′-most end of the cDNA and one from the 3′-most end, just upstream of the polyA addition signal (primers 1 and 9rc, respectively—sequences are given below, and underlined in Fig. 1) were used in PCR to amplify the cosmid template with the Extend long template kit (Boehringer Mannheim, Mannheim, Germany). A 10–11 kb product was amplified. Second, the entire sequence of the subcloned 3.7 kb *Bam*HI/*Eco*RI fragment was determined. The S1 nuclease analysis had defined the 5′-border of exon 1, so this sequencing determined that the 3.7 kb fragment contained approx. 0.95 kb of 5′-flanking sequence. By comparison with the cDNA sequence it also defined the 3′-border of exon 1. The remaining 2.65 kb of 3′-sequence consisted of the beginning of intron 1. Primers 1 and 2rc (underlined in Fig. 1), from exons 1 and 2, were used in PCR with the cosmid clone as template to determine the length of intron 1; sequencing with 2rc defined the sequence at the 3′-end of intron 1, bordering exon 2. The presence of the remaining introns was generally determined by using primer pairs that amplified larger products with cosmid template compared with cDNA template, as assayed on 1 or 2% agarose gels (FMC Corp., Rockland, ME, USA). Intron sizes were estimated by subtracting the genomic product size from the cDNA product size. To define the size of intron 2, a PCR with primers 2 and 3rc was performed; sequencing with these primers determined the intron 2 borders. The same strategy was used with primers 3+4rc for intron 3, 4+5rc for intron 4, and 7+8rc for intron 7. The borders of introns 5 and 6 were more difficult to find. A PCR of the cosmid with primers 5 and 7rc indicated the presence of at least one intron. Sequencing with 7rc defined the 3′-border of intron 6. However, sequencing with primer 5 produced only exonic sequence. Therefore, the 5+7rc PCR product was subcloned into pCRII (Invitrogen). A traditional step-wise sequencing strategy was employed, using the sequence generated by primer 5 to determine what primer (primer 6) to use in the next sequence reaction, and so on. In this way sequencing proceeded through exon 5, intron 5, exon 6, and into the beginning (5′-border) of intron 6 (the primers used to sequence internal regions of intron 5 are not listed as they are not present in Fig. 1). Summation of the determined intron sizes combined with those of the exons yielded the total gene size, 10.8 kb.

Primer sequences (underlined and labeled by a circled number in Fig. 1): Primer 1 = nucleotides 32–55 (as

probes 1 and 2 is protected. Lane 11 shows that the negative control probe is not protected at all by the mRNA. Lane 14 shows that the 297 bp fragment from the interior of the cDNA is protected as expected. (C) Primer extension analysis. Lane 1, radioactively labeled ϕX174+*Hae*III marker; lane 2, kit control primer extension showing the expected 87 nucleotide product reverse transcribed from control mRNA; lane 3, a primer extension reaction using the 25 nucleotide EMMPRIN antisense primer, but no mRNA in the reaction mix; lane 4, a primer extension reaction containing 1 μg of LX-1 mRNA with the 25 nucleotide EMMPRIN antisense primer. A product of 70 nucleotides was produced (arrow). Since the 3′-end of the primer is 33 nucleotides from the A residue of the ATG start codon, the remaining product represents the 5′-UTR (=37 bases).

numbered in Fig. 1) (GGAATCATGGCGGCTGCGCTGTTC); primer 2 = nucleotides encoding amino acid residues 30–35 (GTAGAAGACCTTGGCT); primer 3 = the nucleotides encoding amino acid residues 83–89 (GGAGAGTACTCCTGCGTCTTC); primer 4 = nucleotides (nts) encoding amino acid residues 126–134 (CAAGTCAGAGTCCGTGCCACCTGTC); primer 5 = nts encoding amino acid residues 158–164 (CTTCGTGAGTTCCTCGCAG); primer 6 = nts encoding amino acid residues 258–264 (GACAAAGGCAAGAACGTCC); primer 8 is toward the start of the 3′-untranslated region (see Fig. 1) (GGAGTCCACTCCCAGTGCTTGCAAGATTCC); where the 'rc' designation follows primers 2–8, the sequence is the reverse complement of the sequence given above for that primer; primer 9rc is the reverse complement of nucleotides at the end of the 3′-UTR. Its 5′- to 3′-rc sequence is GAGGTGGGGGCGATCTTTATTGTGGCGGTG.

2.4. 5′-flanking region of the gene

The 3.7 kb *Eco*RI/*Bam*HI genomic fragment isolated from the cosmid clone contained approx. 0.95 kb of sequence upstream of the transcription start site, as determined by sequence analysis. This region was both visually inspected for cis elements and searched for transcription factor binding sites using the Transfac program. Only sequences that conform to the consensus are indicated in the reported sequence.

2.5. Accession numbers

All sequences in Fig. 1 have been submitted to Genbank. They have been assigned accession numbers AF042848, AF042849, AF042850, AF042851, AF042852, AF042853, AF042854, and AF042855.

3. Results

3.1. The EMMPRIN gene clone

A cosmid genomic library was screened with full-length EMMPRIN cDNA to isolate candidate genomic clones. To avoid any possibility of analyzing an artifact cosmid clone, i.e. one generated by ligation of more than one non-contiguous piece of genomic DNA, two criteria were used to assess selected candidates. First, limited sequence analysis revealed that EMMPRIN sequences were contained within the selected positive clone. Second, fluorescent in situ hybridization was used to map the cosmid clone to the correct location on chromosome 19. The metaphase chromosomes of 49 lymphocyte cells were analyzed with the candidate cosmid DNA. Forty-five hybridization signals localized to chromosome 19p13.3, the known location for the EMMPRIN gene (Kaname et al., 1993). No other reproducible hybridization signals were seen. Therefore, the cosmid's genomic DNA represents a single, continuous approx. 30 kb piece of DNA containing the EMMPRIN gene. A 3.7 kb *Eco*RI/*Bam*HI fragment contained the 5′-portion of the gene. PCR amplifications generated the remainder. The genomic primary structure is shown in Fig. 1.

3.2. Determination of the transcription start site

Northern analysis suggested that the full-length mRNA was approx. 1.6 kb. Our cDNA (Biswas et al., 1995) was slightly smaller, missing about 100 nucleotides from the 5′-end. We therefore hybridized fragments of the genomic clone to LX-1 tumor cell mRNA, the source of our EMMPRIN cDNA, and performed an S1 nuclease analysis, to determine the possible transcription start site of the gene, defining the beginning of exon 1. To accomplish this, two genomic fragments of 492 and 331 bp, with identical 3′-ends (at +68 in Fig. 1) were labeled, hybridized to mRNA, and treated with S1 nuclease. As can be seen in Fig. 2B, lanes 5 and 8, 68 bp of both probes were protected. Since the 3′-end of both probes encompasses 31 bases of translated sequence, the total length of the 5′-UTR is 68 minus 31 bases, or 37 bases. No other transcription start sites were suggested by the S1 nuclease analysis.

If the 5′-UTR of the EMMPRIN mRNA was encoded by more than one exon, using a gene fragment as a probe could define an exon/intron junction rather than the true start of exon 1. To rule out this possibility, primer extension analysis was performed. An EMMPRIN-specific antisense primer (reverse complement of nucleotides 46–70 in Fig. 1) was used to reverse transcribe LX-1 cell mRNA. The resulting EMMPRIN cDNA was 70 nucleotides in length (see Fig. 2C). As 33 nucleotides of this product correspond to coding sequence, the remaining 37 correspond to the 5′-UTR. Therefore, both S1 nuclease and primer extension analyses define the start site of transcription (the beginning of exon 1) as that shown by the +1 in Fig. 1.

3.3. The exon/intron structure of the gene

The EMMPRIN gene is encoded by eight exons encompassing 10.8 kb (Figs. 1 and 3). The gene organization is shown in Fig. 3. (The strategy used to arrive at this structure is described in Materials and Methods.) Exon 1 (108 bp) is separated from exon 2 (154 bp) by the approx. 6.5 kb of intron 1, the largest intronic sequence in the EMMPRIN gene. Intron 2 is approx. 700 bp, the second largest intervening sequence in the gene. Exon 3 (83 bp) is separated from exon 4 (138 bp) by the approx. 300 bp intron 3. Intron 4 is approx.

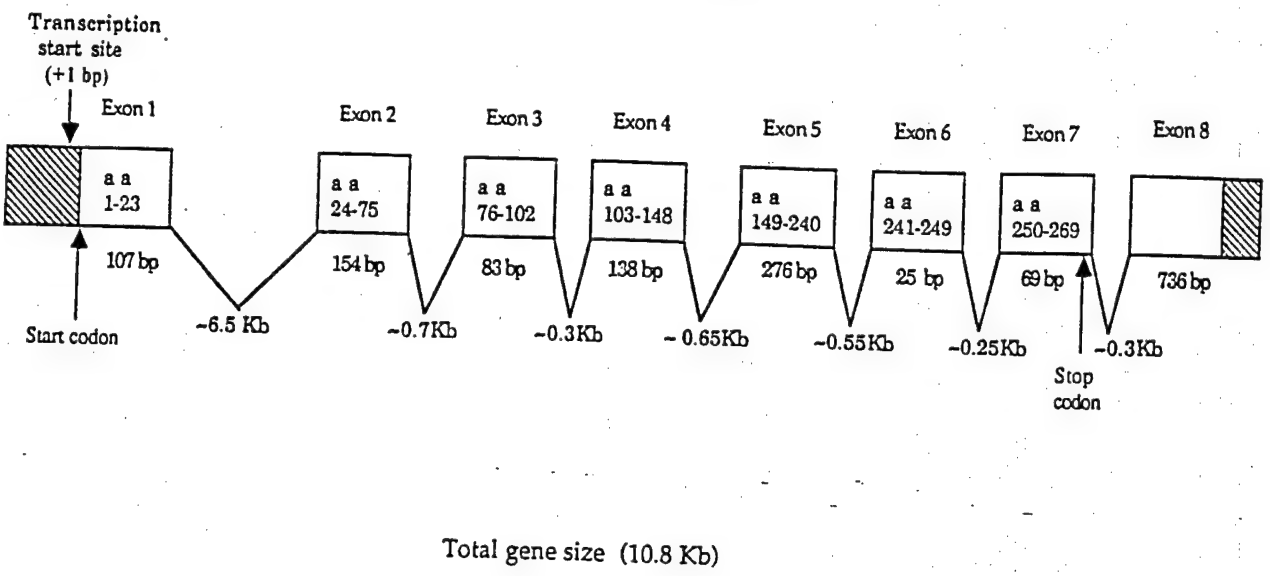

Fig. 3. The EMMPRIN gene organization, outlining the exons and introns. The sum of the exon and intron sizes is 10.8 kb.

650 bp; exon 5 is 276 bp; intron 5 is approx. 550 bp; exon 6 is very short, only 25 bp; intron 6 is approx. 250 bp; exon 7 is 69 bp; intron 7 is approx. 300 bp; and the final exon, exon 8 is 736 bp.

Fig. 4 shows a model of the mRNA, delineating important domains of the protein, and indicating which exons encode these domains. Exon 1 encodes the 5'-untranslated region, the signal peptide and 1 1/3 codons of the first Ig domain. Together, exons 2 and 3 encode the bulk of the first Ig domain: exon 2 encodes 52 codons, or approx. 66% of Ig I; exon 3 encodes 27 codons, or approximately 34% of Ig I. Exons 4 and 5 encode the second Ig domain: exon 4 is 46 codons, comprising approx. 45% of the domain: exon 5 is a 'junctional' exon, encoding the remaining 55% of the Ig domain, as well as the 24 amino acid residues of the transmembrane domain, and a small portion of the intracellular domain. Exons 6 and 7 encode the intracellular domain. Exon 7 also encodes the stop codon and five nucleotide residues of the 3'-untranslated region. Exon 8 encodes the remainder of the 3'-untranslated region.

3.4. The 5'-flanking region of the EMMPRIN gene

Visual inspection of the EMMPRIN gene sequence found no TATA or CAAT boxes in appropriate locations relative to the transcription start site. It did, however, reveal that the transcription start site falls within a CpG island. The occurrence of this dinucleotide is particularly plentiful between nucleotide positions −247 and +6. Transfac computer analysis of the approx. 0.95 kb 5'-flanking sequence revealed the presence of three consensus binding sites for SP1 and two for AP2 (Fig. 5).

4. Discussion

4.1. Comparison of the human exon/intron structure with that of mouse

The gene for mouse EMMPRIN, called basigin, has been localized to chromosome 10 (Simon-Chazottes et al., 1992), and the organization of the mouse gene has been described (Cheng et al., 1994; Miyauchi et al., 1995). The human gene has been mapped by fluorescence in situ hybridization and G-banding on human metaphase chromosomes to 19p13.3 (Kaname et al., 1993), but was not further characterized. Therefore, we decided to isolate and characterize the human EMMPRIN gene structure.

After isolating a cosmid clone containing the gene, we first determined the transcription start site, defining the beginning of exon 1. S1 nuclease analysis revealed that the 5'-UTR of the human EMMPRIN mRNA is 37 bases long. The mouse 5'-UTR was not experimentally determined, but was deduced to be 20 nucleotides from the ATG start codon.

The exon/intron structure of the human gene was determined, and the sizes of the exons and introns were calculated. The overall size of the human gene is larger than the mouse gene. The exons are dispersed in 10.8 kb in the human, and 7.5 kb in the mouse. The sizes of the exons are conserved across the species, therefore the difference in the sizes of the genes is due to smaller introns in the mouse gene. For the most part, the introns for both genes are of ordinary size (700 bp or less) with the exception of the first intron, which is about 4.7 kb in mouse and 6.5 kb in human. Such large first introns are quite common in extracellular matrix genes

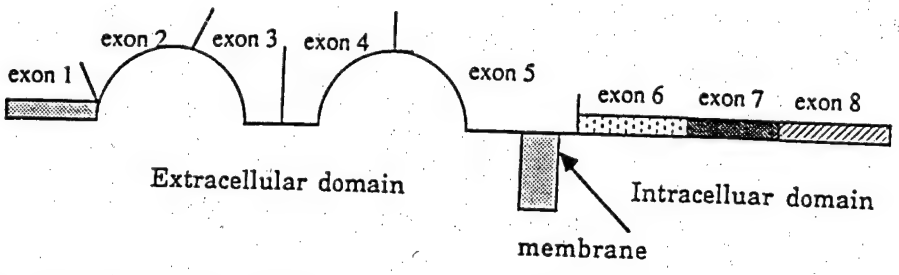

Fig. 4. Schematic diagram of EMMPRIN mRNA, showing exon borders and domain structures. Starting from the left is the 5′-UTR and the signal peptide encoded by exon 1. The two half circles indicate the Ig domains of the extracellular portion of the molecule, showing that each is encoded by two exons. The transmembranous domain is above the cross-hatched grey structure labeled 'membrane'. To the right of the transmembrane domain are the two exons encoding the cytoplasmic domain and one exon (exon 8) encoding most of the 3′-UTR.

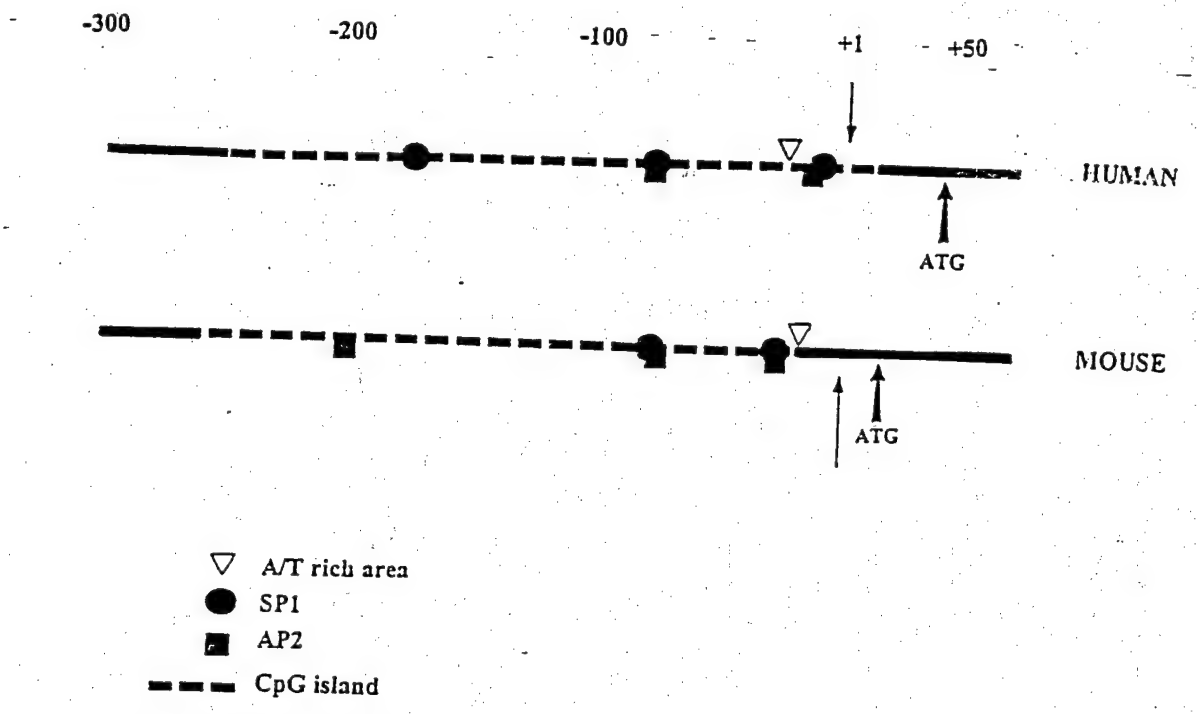

Fig. 5. Schematic of the 5′-flanking regions of the human and mouse EMMPRIN genes. The transcription start sites are aligned and are indicated by thin-based arrows below the +1 designation. The dashed line symbolizes the CpG island. Filled circles denote SP1 binding sites and filled boxes denote AP2 binding sites. The small A/T-rich regions that may facilitate RNA polymerase binding are indicated by open triangles. The ATG start codons are marked with thick-based arrows.

(Takahara et al., 1995; Truter et al., 1992; Yoshioka et al., 1995; Young et al., 1989) and may contain elements responsible for enhancing transcription or for conferring tissue-specific transcriptional regulation.

While the exon structure is highly conserved between human and mouse, some small species differences do exist. The human EMMPRIN gene contains eight exons, one more than the mouse gene. The equivalent of mouse exon 7 is encoded by exons 7 and 8 in the human gene. It is noteworthy that this region corresponds to the 3′-untranslated region of the mRNA. The significance of encoding the 3′-UTR in multiple exons is unknown. Another minor difference is observed at the exon 1/exon 2 border. The last amino acid encoded by human exon 1 is the first amino acid encoded by the mouse exon 2. The final minor difference involves the splitting of codons at the exon/intron borders. In the human gene, all exons begin and end with split codons. In the mouse this is true with the exception of the exon boundaries on both sides of intron 4, where complete 3 nucleotide codons are found.

The human gene, like the mouse gene (Cheng et al., 1994; Miyauchi et al., 1995), has the unusual property that each Ig domain is not encoded by one exon, but by two. Most members of the Ig superfamily encode the Ig domains in a single, unshared exon. Notable exceptions are some of the N-CAM Ig domains and CD4. Miyauchi and coworkers (Miyauchi et al., 1995) have suggested that this splitting of the Ig domains supports the idea that EMMPRIN is closely related to the primor-

dial polypeptide of the Ig superfamily, as also suggested from previously noted homologies with the IgV domain and β chain of MHC class II antigen (Miyauchi et al., 1990, 1991). Also unusual in the mouse and human genes compared with other Ig family members is the fact that the downstream exon of the second Ig domain is a junctional exon, encoding the transmembrane domain and part of the cytoplasmic domain as well. This, however, is a relatively common feature in modular extracellular matrix proteins. For example, junctional exons encoding a portion of a triple helical domain, as well as a portion of a non-collagenous domain, are customary in the collagen genes (Gordon et al., 1989; Sandell and Boyd, 1990; Tikka et al., 1988).

4.2. 5′-flanking region of the EMMPRIN gene

About 950 nucleotides of 5′-flanking sequence of the human EMMPRIN gene were determined and analyzed for consensus binding sites. We compared this region with the comparable region of the mouse basigin promoter, renumbering it to the style presented here for the human promoter. No consensus TATA or CAAT boxes were observed in either the human or mouse gene. An A/T-rich region that may allow access to RNA polymerase exists in the human gene at −21 to −28, and in the mouse gene at −15 to −22 (Fig. 5).

The mouse and human genes both contain consensus binding sites for the transcription factors SP1 and AP2, which are relatively common elements in extracellular matrix gene promoters. In the human sequence three SP1 sites are found (at approx. −175, −80 and −15). Two of these overlap with the two AP2 sites present (approx. −80 and −15). In the mouse three AP2 sites were found (approx. −200, −80 and −25); two of the three overlap the two SP1 sites present (approx. −80 and 25). The conservation of the overlapping AP2 and SP1 binding sites to approx. −80 and the general vicinity of approx. −20 in both species suggests that the position of these factors may be regulatory for the EMMPRIN gene.

The CpG island observed in the human gene between −247 and +6 is conserved in the mouse in an analogous position, −256 to −21. In housekeeping genes, these multiple CG dinucleotides are unmethylated and are thought to play a role in their constitutive expression. Interestingly, CpG islands also appear in the 5′-flanking regions of several extracellular matrix genes, which are not constitutively expressed (Bashir et al., 1989; Corson et al., 1993; Killen et al., 1988; Lee and Greenspan, 1995). The methylation state and the significance of the CpG island is unknown in matrix genes.

Matrix metalloproteinases are necessary for metastasis, yet most members of this enzyme family are produced by stromal cells associated with tumors rather than the tumor cells themselves (Heppner et al., 1996; Hewitt and Dano, 1996; MacDougall and Matrisian, 1995). Fibroblastic production of interstitial collagenase, stromelysin and 72 kDa gelatinase is stimulated by tumor cell-derived EMMPRIN (Guo et al., 1997; Kataoka et al., 1993), providing the most likely explanation for their expression in tumor stromal cells. The levels of expression of EMMPRIN in normal cells are low relative to tumor cells (DeCastro et al., 1996; Muraoka et al., 1993; Polette et al., 1997). Thus, it is likely that carcinogenic compounds or UV light indirectly trigger the up-regulation of the EMMPRIN gene, enhancing tumorigenesis. Elucidating the primary structure of the EMMPRIN promoter is our first step toward studying this process.

Acknowledgement

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EXTRACELLULAR MATRIX METALLOPROTEINASE INDUCER (EMMPRIN) IN BRONCHOPULMONARY AND BREAST LESIONS

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Running title: EMMPRIN in lung and breast tumours.

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Summary

Tumour cells interact with stromal cells via soluble or cell-bound factors stimulating the production of matrix metalloproteinases (MMP), a group of enzymes largely involved in extracellular remodeling in tumour invasion. Among these factors, Extracellular Matrix MetalloProteinase Inducer (EMMPRIN) has been shown to stimulate *in vitro* the fibroblast production of various MMPs such as interstitial collagenase (MMP-1), stromelysin (MMP-3) and gelatinase A (MMP-2). In this study, the EMMPRIN protein was prominently detected by immunohistochemistry in malignant proliferations of the breast and the lung. It was present at the surface of both tumoural epithelial and stromal cells. Since previous studies have demonstrated that stromal cells did not express TCSF mRNAs, it is very likely that EMMPRIN is bind to stromal cells via a specific receptor. Moreover, our observations demonstrated that EMMPRIN is probably shed as membrane vesicles from tumour cells to fibroblasts and endothelial cells. Taken together, our results showed that EMMPRIN is an important factor in tumour progression by causing tumour-associated stromal cells to increase their MMPs production, thus facilitating tumour invasion and neoangiogenesis.

Introduction

Tumour invasion and metastasis are the result of a multistep process including basement membrane disruption, stromal infiltration, intravasation and extravasation, and invasion of a target organ by tumour cells. All these processes require the degradation or remodeling of basement membranes components and of extracellular matrix macromolecules by proteolytic enzymes. Among these proteinases, matrix metalloproteinases (MMPs) are particularly implicated in the metastastic cascade because of their broad spectrum of substrates [1]. *In vivo* observations have demonstrated that in most carcinomas, stromal cells are the principal source of production of MMPs, including interstitial collagenases, stromelysins, gelatinases and membrane-type MMPs [2-10]. Even though quiescent fibroblasts generally produce relatively low amounts of MMPs [11], it is likely that tumour-associated fibroblasts are stimulated to produce the elevated levels of MMPs usually present in malignant tumours.

Tumour cells may interact with stromal cells via soluble or cell-bound factors, stimulating MMP production. Among these factors, a tumour collagenase stimulatory factor (TCSF), recently renamed Extracellular Matrix MetalloProteinase Inducer (EMMPRIN) has been originally isolated and characterized from LX-1 human pulmonary carcinoma cell line[11, 12]. EMMPRIN is a transmembrane glycoprotein of 58 kDa and has been identified as a member of the immunoglobulin superfamily [13]. EMMPRIN has been shown to stimulate fibroblast production of interstitial collagenase (MMP-1), stromelysin (MMP-3) and gelatinase A (MMP-2) [14, 15]. EMMPRIN was observed in normal tissues such as epidermis, retinal pigment epithelium, breast lobules and ductules suggesting that EMMPRIN may have a physiological role in tissue remodeling by causing induction of stromal MMPs [16-19]. However, EMMPRIN expression was prominently found in malignant proliferations. EMMPRIN protein was detected by immunohistochemistry in epithelial tumour cells of bladder and breast carcinomas [19, 20]. Futhermore, EMMPRIN transcripts were expressed by tumour cells of lung and breast carcinomas whereas these mRNAs expression were undetectable or very weak in normal tissues and benign lesions [21]. Taken together, all these data suggest that EMMPRIN participate to tumour progression by stimulating the synthesis of some MMPs by peritumoural fibroblasts. Since previous immunohistochemical studies employing a monoclonal antibody directed against

EMMPRIN (E11F4) have shown that this factor is localized to the outer surface of several cancer cell lines [11, 20], it seems likely that a tumour cell-stromal cell contact is necessary for the EMMPRIN-mediated regulation of MMPs. However, the mecanism of action of EMMPRIN *in vivo* is still unclear.

This prompted us to examine by immunohistochemistry the distribution of EMMPRIN and MMP-2 in regard to the persistance of intact basement membrane which could be an obstacle for the MMP induction by cell-cell contact in various normal, benign and malignant proliferations of the breast and the lung.

Materal and methods

Source of tissue

Tissues were obtained from 20 lungs resected for squamous cell carcinoma (15 cases) and adenocarcinoma (5 cases, including 1 bronchioloalveolar carcinoma), from 2 normal bronchi, from 10 ductal invasive breast carcinomas (1 of grade 1, 7 of grade 2 and 2 of grade 3, according to the Scarf and Bloom classification) and from 3 fibroadenomas of the breast.

Immunohstochemistry

Fresh samples were frozen in liquid nitrogen, cut at -20°C in a Reichert-Jung 2800 Frigocut cryostat (Germany) at a thickness of 5-8 µm and transferred onto gelatin-coated slides. They were washed in phosphate-buffered-saline (PBS) (2 washes, 5 min each) and incubated with specific antibodies using an indirect immunofluorescence technique. In a first step, non specific binding was blocked with 3 per cent bovine serum albumin (BSA; w/v) in PBS for 30 min. Serial sections were then incubated overnight at 4°C in a moist chamber with the monoclonal antibodies against EMMPRIN (G6.2 at a concentration of 10 µg/ml, Chemicon, Temecula), and MMP-2 (CA 406 at a concentration of 2.5 µg/ml, Oncologix, Gaitherburg) . After two washes in PBS for 5 min and one wash in PBS-BSA for 5 min, the sections were incubated with anti-mouse Ig biotinylated complex (Amersham International, U.K.) at a 1: 50 dilution in PBS-BSA solution for 60 min. Then sections were treated by streptavidin-

fluorescein isothiocyanate (FITC) (Amersham International, U.K.) at a 1: 50 dilution in PBS for 30 min.

A double immunostaining was performed for the simultaneous localization of EMMPRIN and type IV collagen. For this double immunostaing, two sucessive labelling reactions for EMMPRIN and type IV collagen were chained up as follows: (i) detection of EMMPRIN using the monoclonal antibody G6.2 at at a concentration of 10 µg/ml in PBS-BSA solution; (ii) F(ab')2-fragments anti-mouse Ig-digoxigenin (Boehringer Mannheim Biochemical, Germany) at a concentration of 4mg/ml in PBS solution; (iii) anti-digoxigenin fluorescein Fab fragments (Boehringer Mannheim Biochemical, Germany) at a concentration of 1.3 mg/ml in PBS-BSA solution; (iv) detection of type IV collagen using a rabbit biotinylated polyclonal antibody (Institut Pasteur, France) diluted 1:1000 in PBS-BSA solution; (v) streptavidin-Texas Red conjugate (Amesham, U.K.) at a 1:50 dilution in PBS.

We tested the absence of cross-reactivity either by omitting the incubation step with the primary antibody, or by replacing the primary antibody with a non-immune IgG. The sections were counterstained with Harris hematoxylin solution for 10 s., mounted in Citifluor antifading solution (Agar, U.K.) and observed with an Axiophot microscope (Zeiss, Germany) using epifluorescence for conventional microscopy or under a confocal laser scanning microscope (Biorad MRC 600).

Results

EMMPRIN localisation

In all lung and breast samples studied, EMMPRIN was detected in all normal and tumoural epithelial cells (figures 1 and 2). In the normal ducts and lobules and in fibroadenomas of the breast, EMMPRIN was frequently confined at the apical cell membrane (figure 1). In the same way, in the normal bronchi, there was an accumulation of EMMPRIN at the apical pole of the ciliated cells, as confirmed by confocal microscopy examination (figure 1). In these normal or benign conditions, EMMPRIN was also detected as a weak labelling at the basal pole of the epithelial cells. In tumoural clusters, EMMPRIN was highly expressed in

all malignant cells with a more intense staining on the cells located at the periphery of the well-differenciated nests (figure 2). The positivities were distributed at the outer cell membrane of these cancer cells with a punctiform pattern (figure 2). Furthermore, EMMPRIN was also found in some stromal cells in breast and lung carcinomas close to tumour cells (figure 2). There were positivities at the cell surface of isolated elongated cells considered as fibroblasts. Moreover, some endothelial cells displayed spotty labellings on their cell membrane (figure 2). In these carcinomas, there were also fine positive vesicles in the connective tissue close to tumour cells suggesting that EMMPRIN could be secreted by tumour cells. Since our previous data have shown no EMMPRIN mRNAs in peritumoural stromal cells, these observations support the hypothesis that these cells would bind the EMMPRIN produced by epithelial tumour cells.

Colocalisation of EMMPRIN and type IV collagen

We next investigated whether basement membrane integrity could be an obstacle to the diffusion of EMMPRIN. Using double labelling of immunofluorescence, we found that the stromal positivities of EMMPRIN were not necessarily associated to the absence of type IV collagen around tumour clusters suggesting that basement membrane integrity is not a limiting factor to EMMPRIN diffusion (figure 2).

Colocalisation of EMMPRIN and MMP-2

Since EMMPRIN has been shown to stimulate the fibroblastic production of several MMPs, we looked at MMP-2 localisation in relation with that of EMMPRIN. As expected, MMP-2 protein was detected in the benign and malignant lesions whereas this enzyme was rarely detected in normal tissues adjacent to tumours. In the carcinomas, this MMP was found both in stromal and tumour cells whereas in fibroadenomas it was only present in some sparse fibroblasts at distance from the proliferating ducts. In all carcinomas, we observed that the presence of EMMPRIN in/on stromal cells was concomitant with the detection of MMP-2 in the same cells (figure 3).

Discussion

This immunohistochemical study demonstrates an epithelial expression of EMMPRIN in normal tissues and in various benign and malignant proliferations of the breast and the lung. These data are in agreement with previous reports using a different antibody (E11F4) in the mammary gland [18]. With this E11F4 antibody, De Castro *et al* [16] have found that normal keratinocytes express EMMPRIN *in vitro* and *in vivo* . In contrast, Muraoka *et al* [20] have detected the presence of EMMPRIN mostly in urothelial malignant cells and considered that this cell surface protein could be a tumour marker for bladder cancers. There is also an apparent discrepancy between our immunohistochemical data and our first observations with *in situ* hybridization on the same kind of tumours. Indeed, EMMPRIN transcripts have been detected only in malignant tumour cells but never in the normal tissues and benign proliferations [21]. Nevertheless, in these latter samples, Northern blot analysis has revealed some weak positivities which could reflect a low transcriptional activity. Thus, the immunohistochemical detection of the protein EMMPRIN in normal epithelial cells may correspond to a low basal level of expression and/or a short half-life of EMMPRIN mRNAs in normal tissues and benign lesions undetectable with *in situ* hybridization. Even though, the EMMPRIN expression in normal epithelial cells was weak, it might be representative of a physiological role in tissue remodeling under particular conditions. In contrast, its large expression in invasive tumour cells of the breast and lung carcinomas clearly support its implication in tumour invasion.

Besides this distribution of EMMPRIN on cancer cells, we also found this protein in peritumoural stromal cells in malignant lesions. Since the biosynthesis of EMMPRIN by fibroblasts has never been reported in previous *in vitro* and *in vivo* studies [11, 19-21], it is very likely that these peritumoural stimulated cells are able to bind the EMMPRIN secreted by epithelial cancer cells. The absence of EMMPRIN protein both in fibroblasts and endothelial cells of normal mammary and lung tissues suggest that peritumoural stromal cells would have acquired the ability to express specific receptors for EMMPRIN during tumour progression. The binding of EMMPRIN to stromal cells could then induce their MMP production. Indeed, we and others have previously showed that stromal cells are the principal source of several MMPs such as MMP-2. We observed in this study that the presence of EMMPRIN is

frequently associated with the expression of this MMP in tumours. Indeed, in our comparative study using serial sections, we detected a concomitant expression of EMMPRIN and MMP-2 in tumoural and stromal cells respectively. Furthermore, we have clearly observed a colocalisation of EMMPRIN protein and MMP-2 in tumoural-associated fibroblasts. So, the EMMPRIN molecule expressed by cancer cells could stimulate the MMPs stromal producton via specific receptors.

Even though, the cellular mechanism of action of EMMPRIN is not yet well understood, several hypothesis could be drawn from our results. In a general manner, it seems that its plasma membrane localization serves to restrict its bioactivity to cells in close proximity. Indeed, EMMPRIN attached to the plasma membrane via a transmembrane domain could interact with a receptor present on peritumoural stromal cells via an extracellular domain. In this condition, the presence and/or the persistence of a selective basement membrane material would represent an obstacle for the direct stimulating effect of EMMPRIN on fibroblasts adjacent to epithelial cells. This may partly explain the absence of concomitant expression of MMPs in normal tissues and their limited expression in benign lesions. This could also reflect the unability of stromal cells in normal tissues to express specific receptors and consequently to respond to EMMPRIN by an increased MMP production. Independently on the presence of an intact BM around tumour clusters, some invasive tumour cells seemed to acquiere the capacity to shed EMMPRIN as components of membrane vesicles, thereby enabling EMMPRIN activity to extend beyond the direct cell contact effect. We, indeed, found in our malignant tumours the presence of fine positive vesicles in the connective tissue close to tumour cells, even in the presence of type IV collagen deposited between tumour clusters and the stroma. This "secreted" form of EMMPRIN could therefore bind to specific receptors present only in peritumoral stromal cells and stimulate them to produce MMPs in carcinomas.

In conclusion, our results describing an intense expression of EMMPRIN in cancer tissue clearly implicate EMMPRIN in tumour invasion. The detection of EMMPRIN protein on peritumoural stromal cells and in fine vesicules in the connective tissue also support the hypothesis that this factor could be shed from tumour cells and fixed by tumour-associated stromal cells. This would further stimulate the stromal production of MMPs facilitating tumour

invasion and neoangiogenesis. Taken together these results therefore advances our understanding of the cooperation between cancer and host cells during the invasion and metastatic process.

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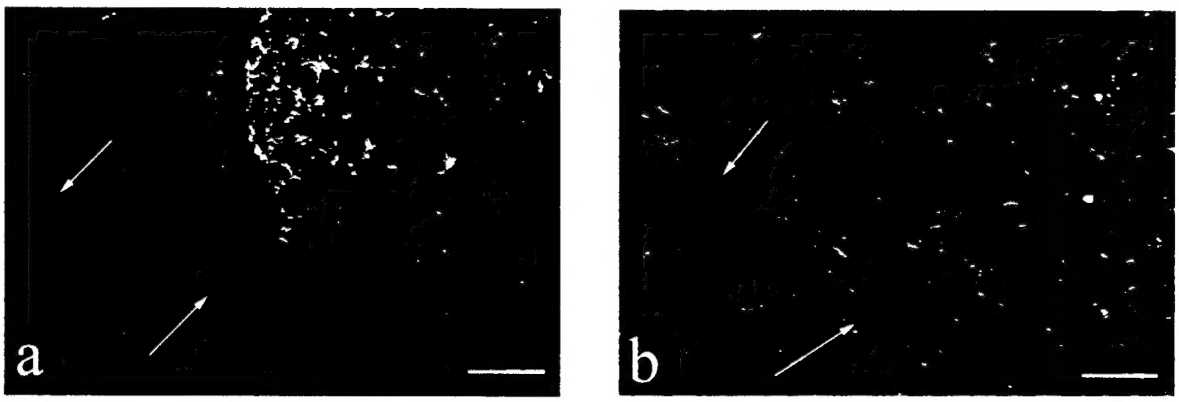
Legends of figures

Figure 1: EMMPRIN localisation in normal and benign proliferations of breast and lung carcinomas. (a) EMMPRIN is present on the outer membrane of epithelial cell with a dense labelling at the apical cell membrane (arrows) in normal lobule breast (Bar =). (b) In fibroadenoma of the breast, EMMPRIN was also prominently found at the apical pole of the epithelial cells by confocal microscopy analysis (arrows) (Bar =). (c) By confocal microscopy analysis, EMMPRIN was distributed at the apical surface of the epithelial cells in normal lung (arrows) (Bar =) (d)

Figure 2 : EMMPRIN detection in breast and lung carcinomas. (a)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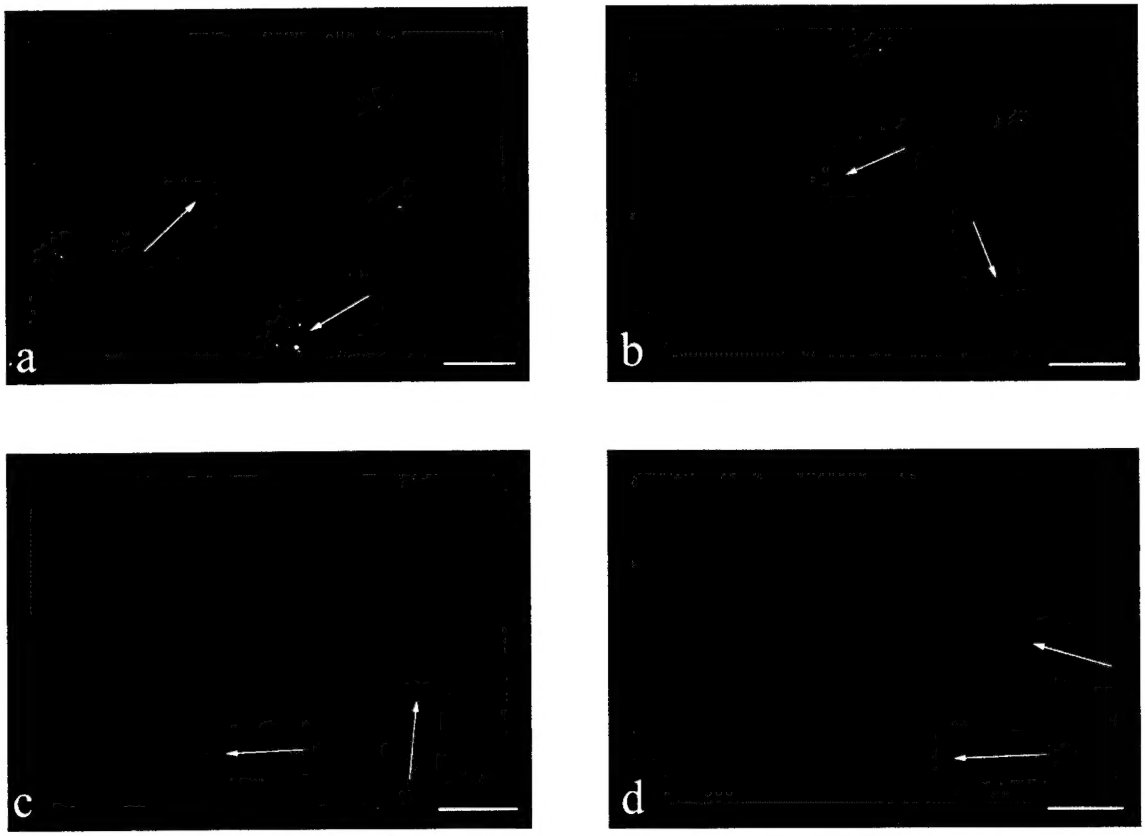
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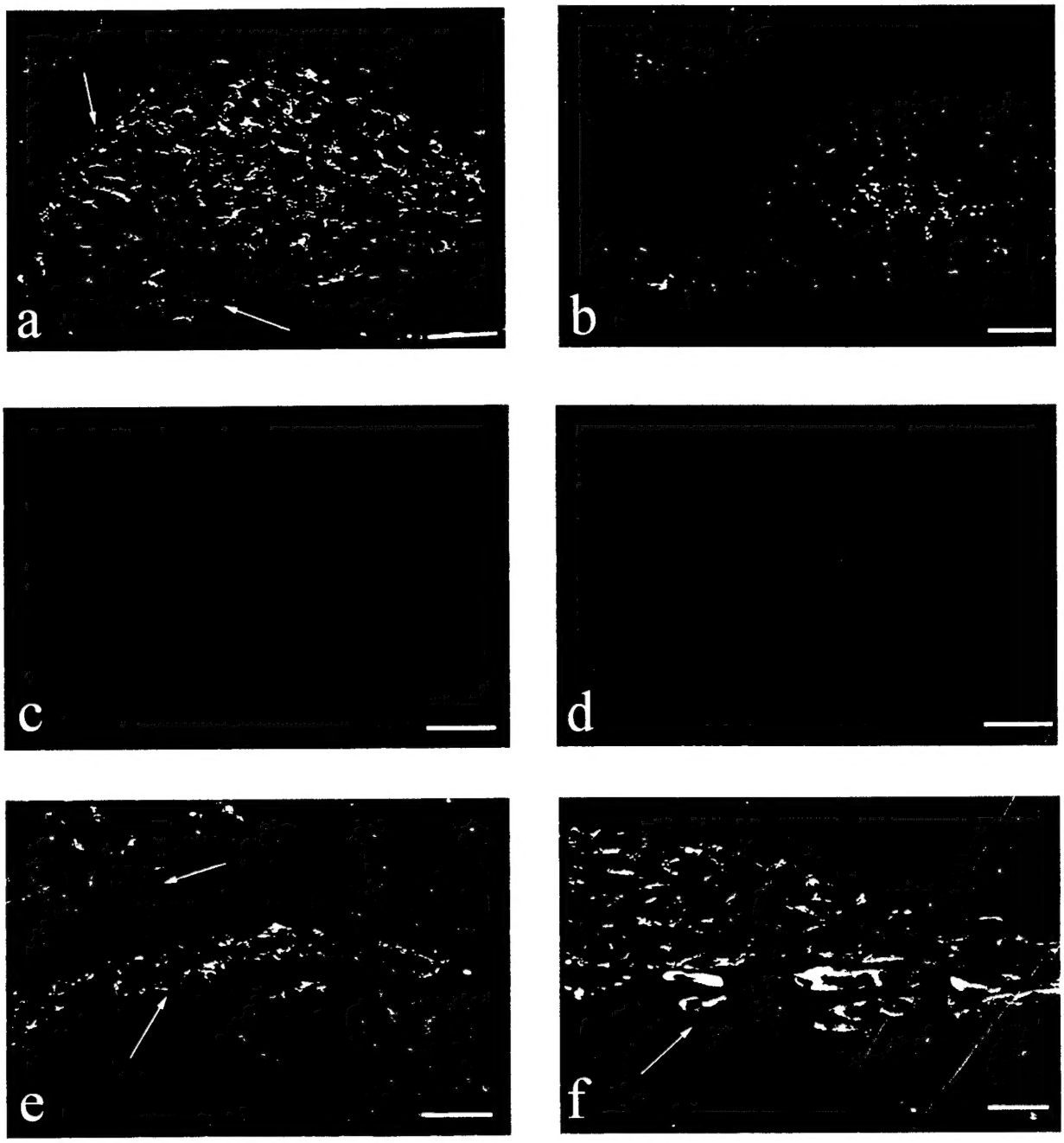
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correlated with TIMP-1 measured in EDTA plasma (mean 73.5 ± 14.2 µg/L) from the same individuals in a set of 100 healthy blood donors (Spearman's Rho = 0.62, $p < 0.0001$). The mean level of TIMP-1 in citrate plasma from 19 patients with advanced breast cancer was 292 ± 331 µg/L and a Mann-Whitney test demonstrated a highly significant difference between TIMP-1 levels in healthy female blood donors and breast cancer patients ($p < 0.0001$). Similar findings were obtained for 143 patients with advanced colorectal cancer (mean: 240 ±145 µg/L). The results show that TIMP-1 is readily measured in plasma samples by ELISA and that increased levels of TIMP-1 are found in patients with advanced cancer. It is proposed that plasma measurements of TIMP-1 may have value in the management of cancer patients.

PB7.24
Extracellular matrix metalloproteinase inducer (EMMPRIN) facilitates tumor angiogenesis
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We have demonstrated that EMMPRIN a 58 kDa membrane bound glycoprotein produced by cancer cells, stimulates peritumoral fibroblast production of MMPs hence enhancing cancer invasion (J Biol Chem 272: 24, 1997). In this study we have compared the effects of EMMPRIN (purified from LX-1 cancer cells) and recombinant VEGF on human umbilical vein endothelial cell (HUVEC) production of MMPs and TIMPS. Confluent HUVEC were incubated in serum-free media for 4–24 hours with 1–100 nM EMMPRIN or VEGF. Conditioned media was collected and the concentration of MMPs and TIMPs were determined by ELISAs and gelatin substrate zymography. EMMPRIN increased MMP-1, MMP-2, MMP-3 and MT1-MMP production and secretion by 30–500%; TIMP-1 secretion was enhanced by 60%. VEGF had a greater stimulatory effect on MMP-1 and TIMP-1, but had a weaker effect on MMP2 and MMP-3 than EMMPRIN. We conclude that EMMPRIN plays a role in the early phase of tumor angiogenesis by inducing the degradation of endothelial basement membrane.

PB7.25
Quantitation of the complex between urokinase plasminogen activator and its type-1 inhibitor in plasma of healthy donors and breast cancer patients
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The amount of the complex between urokinase plasminogen activator (uPA) and its type-1 inhibitor (PAI-1) in a biological sample is proposed to reflect the ongoing level of proteolytic activity. We now report the first kinetic ELISA suitable for the specific quantitation of uPA:PAI-1 complex in plasma of healthy donors and breast cancer patients. The assay consists of a sandwich format with monoclonal capture antibodies against uPA and polyclonal detector antibodies against PAI-I. A purified standard of uPA:PAI-1 complex added to plasma can be specifically measured in the assay with a detection limit below 2 pg/ml, and neither free uPA nor free PAI-1 are detected by the assay. This ELISA was applied to citrate plasma from 19 individual patients with advanced breast cancer and 39 age-matched healthy females. The median plasma level of complex for breast cancer patients was 68 pg/ml, range < detection limit to 8700 pg/ml, whereas for the healthy females the median value was below the detection limit, range < detection limit to 200 pg/ml. For the whole set a significant difference between complex levels in plasma from healthy females and breast cancer patients was found (Wilcoxon test: $p = 0.0001$). For the patients, plasma uPA:PAI-1 content, respectively, and the complex was found to represent a variable, minor fraction of the total uPA and PAI-1 contents in each sample. Thus the data is consistent with release of uPA:PAI-1 complex from tumor tissue into blood, and the presented ELISA is found well suited for quantitation of this complex in plasma of breast cancer patients.

PB7.26
Correlation between matrix metalloproteinases/tissue inhibitors of metalloproteinases and invasive/metastatic potential of human cancer cells
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In order to study the correlation between matrix metalloproteinases (MMPs)/tissue inhibitors of metalloproteinase (TIMPs) and invasive/metastatic potential of cancer cells, the expression of six MMPs and two TIMPs was investigated in three groups, eight kinds of human cancer cell lines with variant invasive and metastatic potential, by employing the methods of RT-PCR, northern blots, western blots and zymography. In a pair of lung cancer cell lines, the highly invasive metastatic cell line PG secreted much more amount of gelatinase A and gelatinase B (MMP2 and MMP9) than the low invasive/metastatic cell line PAa. However, this correlation between MMP expression and cancer invasive/metastatic potential was not shown so clearly in the other two groups of cell lines. In the four melanoma cell lines, the lowest invasive/metastatic potential cell line WM35 secreted the lowest amount of MMP2, while the second lowest invasive metastatic cell line WM1341B produced the highest levels of activated MMP2 and pro-MMP2. In a pair of prostate cancer cell lines, both the low invasive metastatic potential cell line PC-3 and the high invasive/metastatic potential cell line PC-3M secreted trace of MMP2, and PC-3 expressed a higher level of MMP9. Western blots for TIMP1 and TIMP2 showed that the expression level of TIMP2 protein in WM1341B was the highest in the melanoma cell lines and PC-3 secreted much more TIMP1 than PC-3M. So compared with the mere expression level of MMPs, the relative balance of MMPs and TIMPs may be a more precise index in reflecting the invasive/metastatic potential of cancer cells. The expression level of membrane-type 1 metalloproteinase (MT1-MMP) and mRNA was much higher in the four melanoma cell lines than that in the other cancer cell lines, and activated MMP2 was detected only in these melanoma cell lines. On the other hand, PG, a lung cancer cell line whose MT1-MMP mRNA expression level was quite low, could secrete pro-MMP2, but no activated MMP2 could be detected in its serum-free conditioned media. The close association between MT1-MMP expression level and MMP2 activation support the idea that MT1-MMP could bind and activate pro-MMP2. No expression of matrilysin, stromelysin 1 and membrane-type 2 metalloproteinase could be detected in these cancer cell lines.

PB7.27
The JNK pathway regulates a switch between protease phenotypes in cells without an activated Ras
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We have shown that *ras*-transformed NIH3T3 cells in which both the ERK and JNK activities are elevated have urokinase plasminogen activator (uPA)-dependent mechanism of Matrigel invasion and experimental metastasis (phenotype uPA+/CL-). In contrast, *ras*-transformed cells in which ERK activity was elevated and JNK activity inhibited showed a cathepsin L (CL)-dependent mechanism of invasion (phenotype CL+/uPA-). We now show that these protease phenotypes can be generated in untransformed NIH 3T3 cells that express PDGF receptors and acetylcholine G_q-linked m1 muscarinic receptors which selectively stimulate the ERK or JNK pathways, respectively. Treatment of these cells with PDGF alone induced elevated ERK activity and high levels of CL mRNA.